A STUDY OF THE COMPOSITION OF GRASSES AND FODDER PLANTS WITH SPECIAL REFERENCE TO THE ORGANIC ACID COMPONENTS AND THEIR TRANSFORMATIONS DURING CONSERVATION

-by-

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CONTENTS

I N T R O D U C T I O N

Grasses and other green crops are the natural food for the majority of our livestock, and their importance in agricultural economy is becoming increasingly evident. Before the two world wars, European countries augmented their own grass supplies by importing grain and concentrated food. The aim now however is towards self-sufficiency, and as the need for food is always increasing, it is important to feed our livestock to the maximum upon home-grown foods.

In the higher plants there is found a wide variety of aliphatic and aromatic organic acids (1,2), of which the metabolic importance has only recently been recognised. Chibnall (3a) refers to the organic acids as occupying the central, and therefore the key position in the carbohydrate, protein and fat metabolism of plant cells.

There is a closely related series of acids which are widely distributed and often referred to as the "plant acids". The most fully investigated of these are: malic, citric, succinic, tartaric and oxalic acids. Iso-citric, fumaric, oxaloacetic, cis-aconitic and α-ketoglutaric acids are present in smaller quantities in plants, but are probably of equally wide distribution and importance.

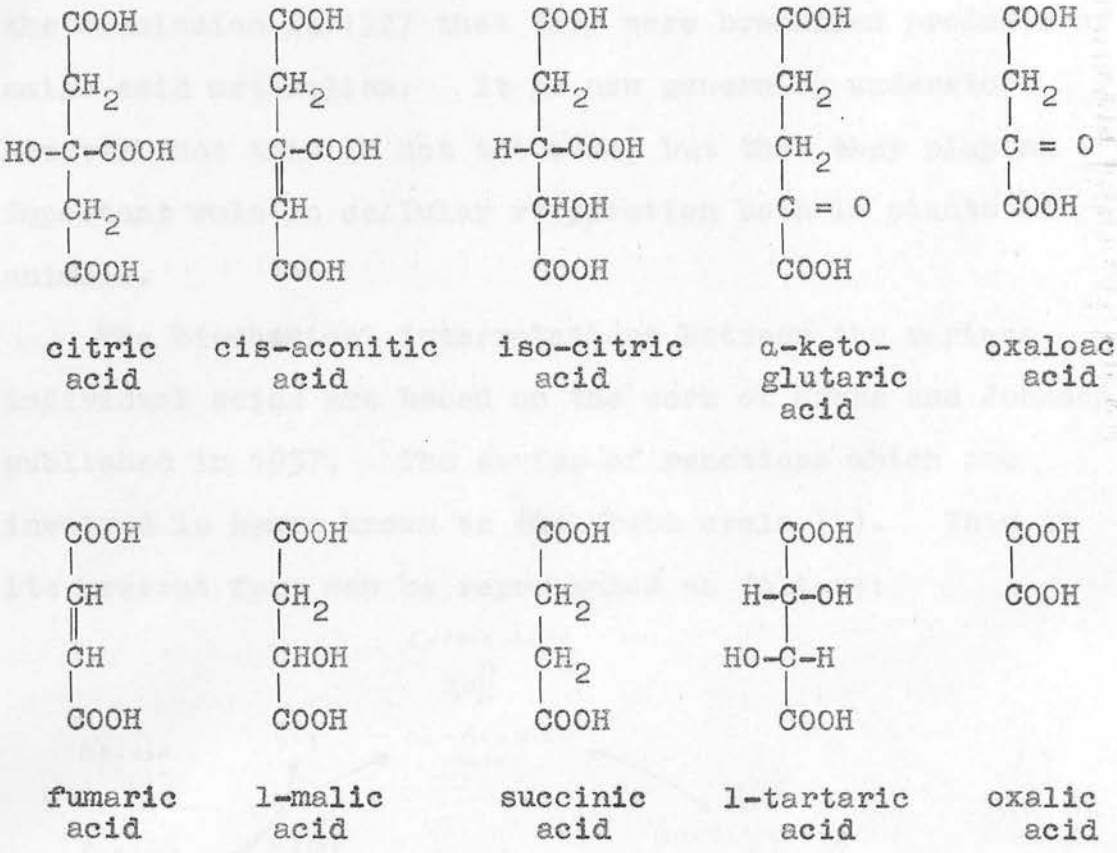


Fig. 1. The plant acids (1a).

The total organic acid content of plants varies from less than 1% to about 30% of the dry weight of plant tissue (1a). They are usually present wholly or partly in the form of salts, combined with metals such as sodium, potassium and calcium, taken up by the roots. A buffer system is thus formed which controls the pH of the cells. Kostytschev (1b), who made a detailed examination of the role of the plant acids, came to

the conclusion in 1927 that they were breakdown products of amino acid metabolism. It is now generally understood however that this is not the case, but that they play an important role in cellular respiration both in plants and animals.

The biochemical interrelations between the various individual acids are based on the work of Krebs and Johnson published in 1937. The series of reactions which are involved is hence known as the Krebs cycle (4). This in its present form can be represented as follows:

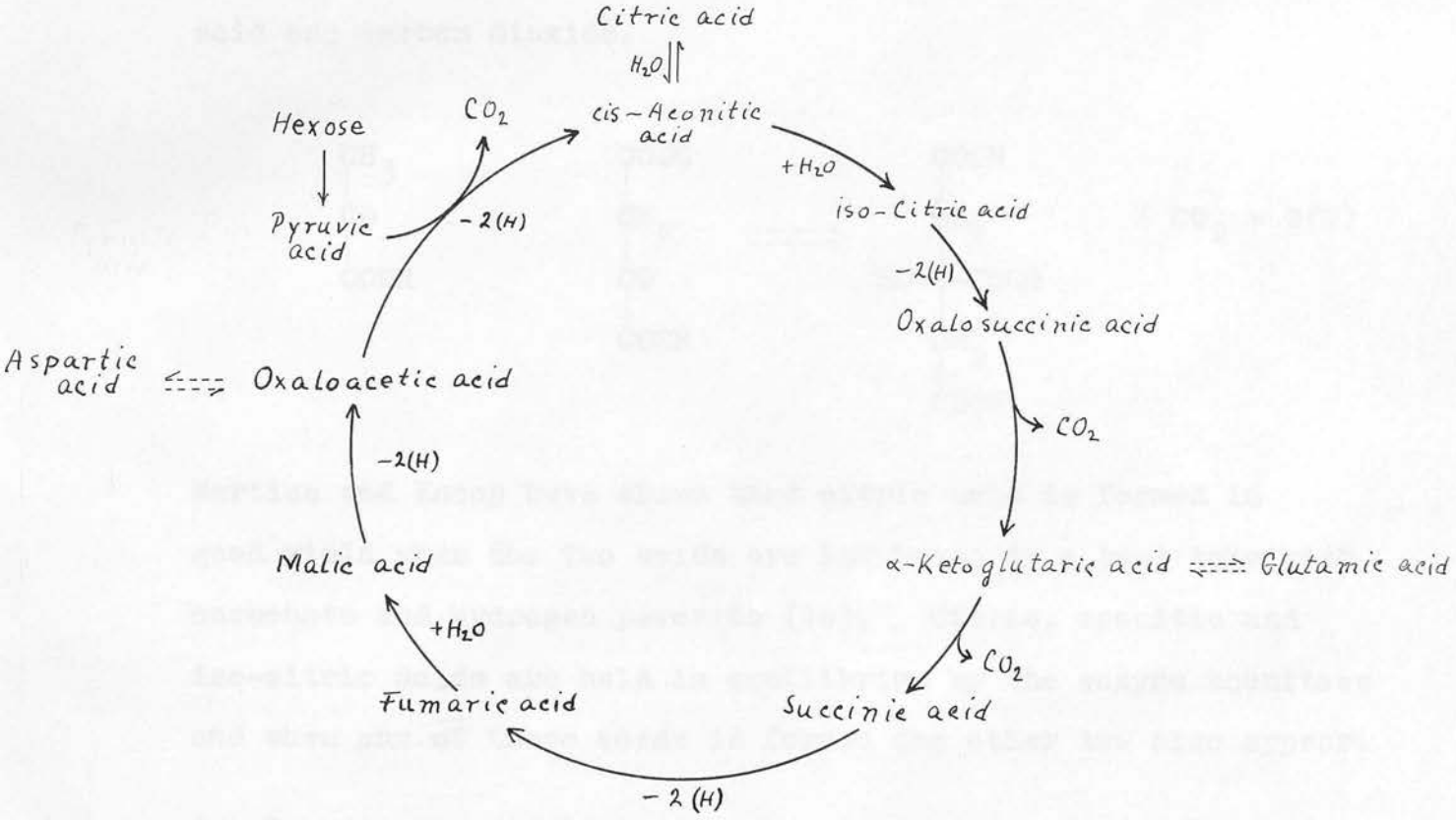
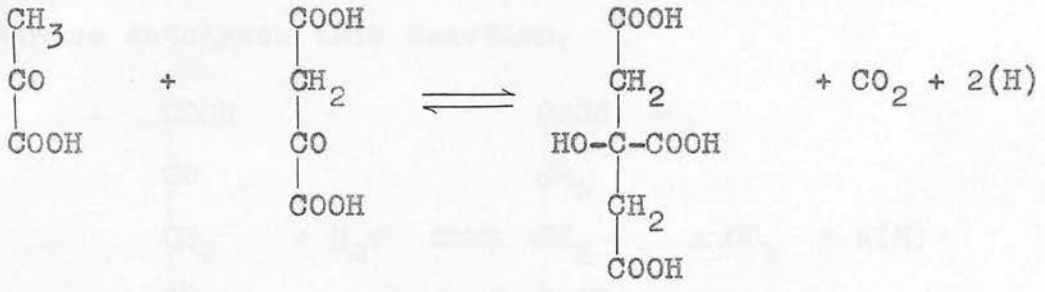


Fig. 2. The Krebs cycle (1c).

In cell respiration the hexoses, which are built up in the plant during photosynthesis, are broken down to pyruvic acid through a number of phosphorylated intermediates (1d, 5). The release of energy in the phosphate bond to other reactions has come to be recognised as an important method by which the respiration energy is made available to energy-consuming processes in the plant.

The following reactions have been found to take place between the acids in Krebs cycle:

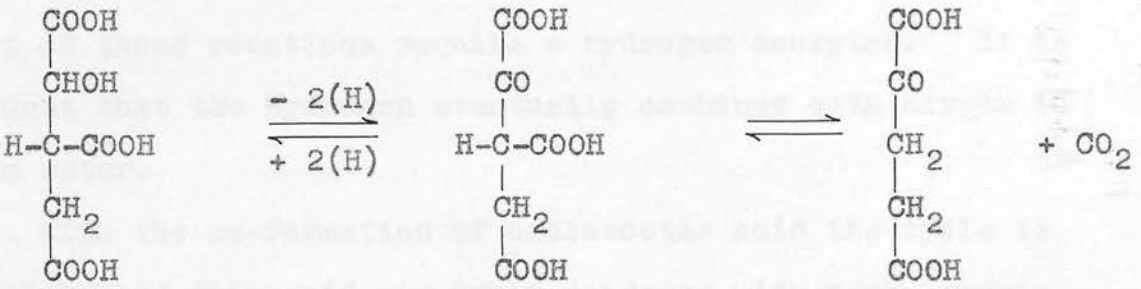
1. Pyruvic acid reacts with oxaloacetic acid to form citric acid and carbon dioxide.



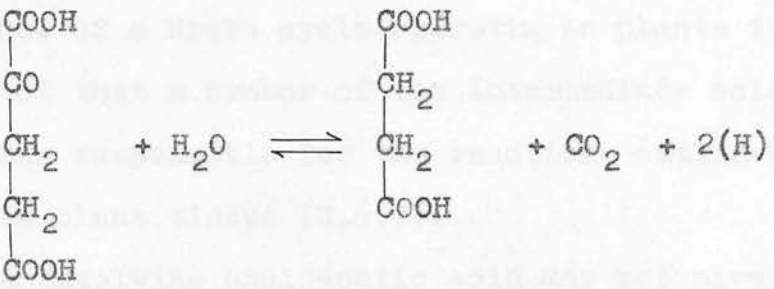
Martius and Knoop have shown that citric acid is formed in good yield when the two acids are incubated in a test tube with carbonate and hydrogen peroxide (1e). Citric, aconitic and iso-citric acids are held in equilibrium by the enzyme aconitase and when one of these acids is formed the other two also appear.

2. Iso-citric acid is oxidised by iso-citric acid dehydrogenase

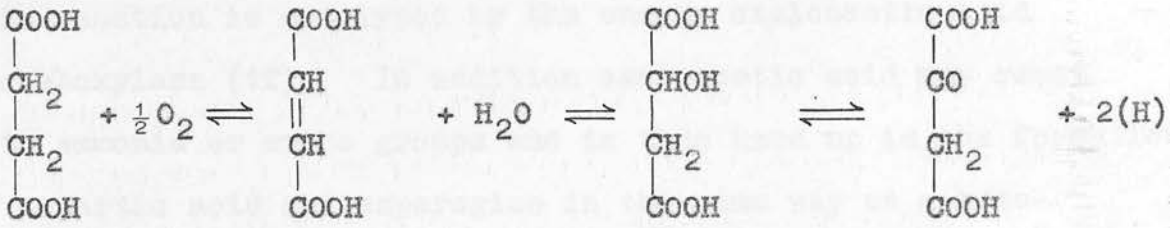
to oxalosuccinic acid, an intermediate, which is rapidly decarboxylated by the enzyme oxalosuccinic carboxylase to form α-ketoglutaric acid.



3. Decarboxylation and oxidation of α-ketoglutaric acid leads to the formation of succinic acid. α-Ketoglutaric dehydrogenase catalyses this reaction.



4. Succinic dehydrogenase catalyses the formation of fumaric acid from succinic acid and vice versa. The oxidation product, fumaric acid, is converted to malic acid by the addition of water. This is catalysed by the enzyme fumarase. Thereafter malic dehydrogenase catalyses the oxidation of malic acid to oxaloacetic acid.

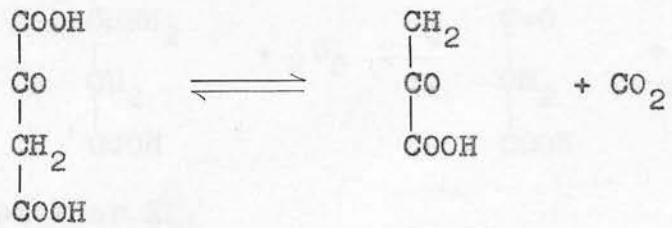


Most of these reactions require a hydrogen acceptor. It is thought that the hydrogen eventually combines with oxygen to form water.

With the re-formation of oxaloacetic acid the cycle is complete and this acid can again condense with more pyruvic acid. Thus in one operation of the cycle there is a loss of a molecule of pyruvic acid with production of 3 molecules of carbon dioxide, and in fact the net result is a breakdown of hexose to carbon dioxide and water.

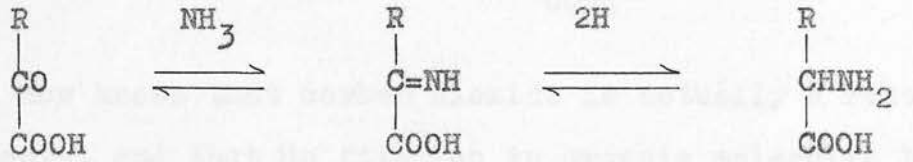
The acceptance of a Krebs cycle operating in plants is based upon the fact that a number of the intermediate acids and all the enzymes responsible for the reactions outlined above are found in plant tissue (2,6,7).

The reaction involving oxaloacetic acid may not always take the course outlined above. It may be removed from the cycle by several reactions, one of which is the breakdown to pyruvic acid and carbon dioxide.



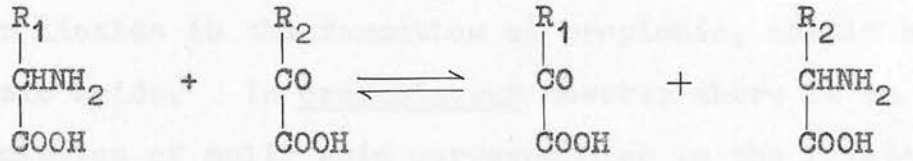
This reaction is catalysed by the enzyme oxaloacetic acid decarboxylase (1f). In addition oxaloacetic acid may react with ammonia or amino groups and is thus used up in the formation of aspartic acid and asparagine in the same way as α-keto-glutaric acid can be converted to glutamic acid.
A simple pattern for these reactions is:

I.



α-keto acid — α-imino acid — α-amino acid

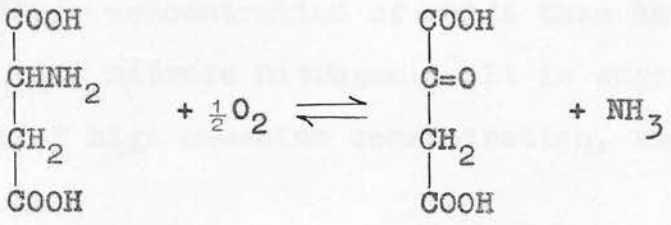
II. (Transamination)



These reactions were first known to occur in animal tissue (8) but were later also demonstrated in plant tissue (1g,3b,3c,8a).

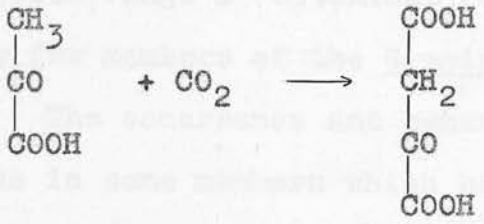
On the other hand oxaloacetic acid may be formed by oxidative deamination (1g) according to the equation,

III.



or by reaction I or II.

Oxaloacetic acid may also be formed by addition of carbon dioxide directly to the pyruvic acid molecule (9), by the Wood and Werkman reaction.



It is now known that carbon dioxide is actually a metabolite in plants, and that its fixation in organic molecules is not restricted to photosynthesis in green plants and to chemosynthesis as carried out by certain groups of micro-organisms. Wood and Werkman (9) found in 1936 that Propionibacterium, when incubated anaerobically with glycerol, utilised carbon dioxide in the formation of propionic, acetic and succinic acids. In crassulacean however there is an accumulation of malic acid corresponding to the fixation of carbon dioxide (10).

The form in which nitrogen is made available is a factor which greatly influences the organic acid content of plants (1h,8a). Plants supplied with nitrogen in the form of ammonia have a lower concentration of acids than have similar plants supplied with nitrate nitrogen. It is suggested (1h) that in the case of high ammonium concentration, the organic

acids are kept at a low concentration by reactions producing amino acids and amides from oxaloacetic and α-ketoglutaric acids.

Past work on the occurrence and distribution of plant acids has covered a wide range of botanical families and species, but relatively few members of the Gramineae have been investigated. The occurrence and behaviour of the most important acids in some members which have been examined are summarised in the following table:

Table 1.

Citric Acid

Plants	Material	Remarks	Ref.
Sugar cane	Juice	Small amounts present. Increase during sprouting.	2
Perennial Rye-grass	Leaf clippings	0.96-0.50% of total acids	2
Perennial Rye-grass, Timothy and Cocksfoot	Whole plant	Decrease with age. Decrease from morning to evening.	11
Cocksfoot	Whole plant	Decrease with age	11
Mixed grass	Whole plant	Present	12
Oats, Barley, Wheat and Rye	Seeds	0.01-0.03% of dry matter Increase during germination	2
Oats, Barley, Wheat and Rye	Whole plant	Present	13, 14
Zea Mays	Seeds and whole plant	Present	2, 13

Malic Acid

Plants	Material	Remarks	Ref.
Wheat and Blue grass	Leaves	8-18 m. equiv. per cent	2
Sugar cane	Juice	Present	2
Perennial Rye-grass	Leaf clippings	3.5-1.2 % of total acid. Decrease with age.	2
Perennial Rye-grass, Cocksfoot and Timothy	Whole plant	Increase from morning to evening	11
Mixed grass	Whole plant	Present	12

Oxalic Acid

Plants	Material	Remarks	Ref.
Sugar cane	Juice	Present	2
Perennial Rye-grass	Leaf clippings	0.31-0.2 % of total acid. Decrease with age.	2
Blue grass and wheat	Leaves	Absent	2
Wheat	Whole plant	0.11 % of dry matter	14
Barley, Maize, Oats and Rye	Whole plant	0.02-0.05% of fresh weight	13

Succinic Acid

Plants	Material	Remarks	Ref.
Sugar cane	Juice	Small amounts	2
Perennial Ryegrass, Timothy and Cocksfoot	Whole plant	Present	11
Mixed grass	Whole plant	Present	12

Aconitic Acid

Plants	Material	Remarks	Ref.
Sugar cane	Juice	Present	2
Barley, Maize, Oats and Rye	Whole plant	Present	13
Wheat	Whole plant	Present	14

As well as the acids mentioned in table 1, Barnett and Duncan (15) found small quantities of acetic acid in fresh grass. Furthermore, quinic, shikimic and chlorogenic acids have been found in mixed grass samples (12, 16).

Changes during crop conservation.

The growth of grass and other fodder crops is restricted by cold and by drought. It is therefore necessary to preserve

the fodder at its most valuable stage for use during these periods. It is essential that the method of conservation is cheap, and the loss of valuable nutrients as low as possible.

Artificial drying of the green crop is an efficient method of conservation, but it has the disadvantage of being very expensive. Haymaking by drying in the field has been used since early times for preserving grass, and is still the method most frequently employed in spite of considerable losses of feeding value.

Apart from mechanical losses in haymaking, there is a considerable loss of dry matter due to cell respiration which continues after the crop has been cut. Losses of the order of 20% of the dry matter may occur under good conditions, while in bad weather losses more than twice as high as this have been reported (17a). Furthermore, the material which is lost is the most digestible part of the plant (17b). It is also obvious that under poor conditions some of the soluble nutrients present may be washed away by rain falling on the hay.

Silage-making involves the tight compaction of fresh fodder into containers in such a way that air is largely excluded. Much research has been done on the process of ensilage during the last century (17,18), and methods have

been developed for the successful ensilage of various crops under different climatic conditions.

Microorganisms are always present on the surface of the crop when it is harvested and these multiply rapidly under favourable conditions. The course taken by this fermentation is very important, as it will decide the value of the final product.

The changes taking place in an ensiled crop are briefly as follows:

When the material is packed into the silo the respiration of the plant cells continues for some time, resulting in breakdown of carbohydrates with the final production of carbon dioxide and water and generation of heat. The air is finally exhausted of its oxygen and an intracellular, or anaerobic respiration or fermentation takes place. In this process the carbohydrates are broken down to alcohol and carbon dioxide without the participation of atmospheric oxygen. This is similar to, and possibly identical with the process known as alcoholic fermentation brought about by yeast (6a). Thus the pyruvic acid formed by the breakdown of hexoses may be reduced to lactic acid or decarboxylated to acetaldehyde, which again is reduced to alcohol (1i,5a).

These changes continue until the enzymic actions cease due to exhaustion of readily available material, or increase

of acidity. At the same time, as a result of the heat generated in the mass, the bacteria are multiplying rapidly, and bacterial changes are starting using the contents of the dead plant cells as a medium.

The most desirable bacterial fermentation is that due to the lactic acid-forming organisms, mainly lactobacilli. The best conditions for growth of lactobacilli are a temperature of 37°C. and a low concentration of oxygen, i.e. they are microaerophilic, and they can tolerate high concentration of acid. If an adequate supply of carbohydrate is available, these organisms, without noticeably affecting the proteins (19), produce lactic acid equivalent to 1-2% of the fresh material, and reduce the pH to 4.2 or less. Below pH 4 lactic acid fermentation is the only noticeable process which occurs (20).

If lactic acid production is insufficient, an undesirable fermentation may set in due to the growth of butyric acid-producing organisms, belonging to the clostridium group (18a). These organisms produce butyric acid as the main product, as well as small amounts of other volatile acids from carbohydrates. They may also decompose the lactic acid already formed, producing mainly butyric acid (17d,20a). In both cases there are produced large amounts of carbon dioxide. The clostridia often decompose proteins by means of their enzymes and large amounts of ammonia may be the result (17e,22).

Their action is almost inhibited at pH 4 or below (20). Otherwise the breakdown of proteins to the stage of amino acids is due to the plant enzymes, a process which also is almost inhibited below pH 4 (17e,23).

The desirable changes taking place in a silage result in the breakdown of carbohydrates with the formation of mainly lactic and acetic acids. Other shortchain fatty acids are produced in small amounts (18d). The feeding value of this portion of the carbohydrate however is not lost, since lactic acid is itself a foodstuff which can be readily utilised by the animals (24). The lower fatty acids are absorbed into the bloodstream and metabolised by the animals (25,26).

Under suitable conditions the lactobacilli are capable of producing sufficient acid to lower the pH to 4.2 or below. Virtanen and his co-workers in Finland showed that the respiration, protein breakdown and butyric acid fermentation were largely prevented at pH 4 (20) and devised the A.I.V.-method, widely used in Scandinavia, whereby the pH is rapidly brought down to the desired value by means of addition of mineral acid.

Usually a rapid fall of pH takes place when the crop has been wilted beforehand. A low moisture content of the ensiled material inhibits the activity of the butyric acid-

forming bacteria (27,28,29).

Fodder of a high protein and a low carbohydrate content, e.g. lucerne and immature grass, is difficult to ensile without pretreatment, as there is insufficient carbohydrate present to permit a rapid lactic acid fermentation. In such cases satisfactory results are obtained by spraying the crop with molasses prior to ensilage, or by mixing into it a carbohydrate-rich fodder. Addition of acids may also result in a good silage.

A more recent additive has been sulphur dioxide, added to the crop either in the gaseous state or as sodium meta-bisulphite, which decomposes to give sodium sulphite and sulphur dioxide. A limited degree of fermentation occurs after a period of months with formation of small amounts of lactic and acetic acids, and no butyric acid (30). Little ammonia is formed, but there is a considerable hydrolysis of protein to amino acids (31).

The temperature in the silo may be controlled to a certain extent by variations in the packing technique. A wet crop, or a crop which has been chopped or crushed makes a very compact silo. The cell respiration is suppressed owing to lack of oxygen and the temperature does not rise above about 20°C. This is called a "cold fermentation" silage and may give a product of good quality.

A "warm fermentation" process with temperatures of the order of 50°C. is obtained with a loosely compacted, stemmy crop. A great deal of air is occluded in the mass, and the cell respiration continues rapidly, causing serious losses. The mass compresses by its own weight, forming a pleasant smelling fodder, but with an impaired protein digestibility (18c).

Usually a good silage is made at 27-38°C., the so-called "low temperature" silage (17f).

Plant acid determination.

The older methods of plant acid determination were laborious and time-consuming. The individual components were separated either by fractional precipitation of the salts, or by fractional distillation of the esterified acid fraction (1j). The application of specific micro methods, introduced by Pucher, Vickery and their colleagues (32,33) led to reliable methods, which could be used for routine analyses of plant tissue.

The fresh tissues were dried at 80°C. in a ventilated oven and ground to a powder. This was mixed thoroughly with 0.5N sulphuric acid, and the organic acids were extracted with ether. Water was added to the ether extract, and the ether was removed

by evaporation. An aqueous extract of the acids was thus obtained. The total organic acid content was determined by potentiometric titration, corrections being made for the fact that a small amount of sulphuric acid was extracted with the ether, that only 50% of the oxalic acid was titrated, and finally that the acid fraction also contained the nitric acid present in the sample. Estimations of the individual acids were then carried out on aliquots of this acid extract.

Oxalic acid was removed by precipitating as calcium oxalate and estimated by titration with permanganate in hot solution. A further aliquot was oxidised with permanganate in the presence of bromide. Under these conditions citric acid gave pentabromo-acetone which could be removed by extraction with petroleum ether. This product was dehalogenated, and the bromide ions produced titrated with silver nitrate. The amount of citric acid could then be calculated. The malic acid present was oxidised to an unknown steam-volatile product which could be precipitated with dinitrophenylhydrazine. The resultant product could be estimated colorimetrically. Succinic acid was estimated by converting to succinyl-p-toluide, which was removed and weighed.

Thus the estimation of the individual organic acids in plants was still no easy task. The problem was simplified considerably, however, by the development of partition

chromatography and ion-exchange techniques.

Partition chromatography on silica gel has been used successfully for separating mixtures of organic acids. This technique was originally devised by Martin and Synge (34) who used water as the stationary phase and chloroform-butanol as mobile phase. The method has been used successfully by Marvel and Rands (35). Isherwood (36) greatly improved the method using dilute sulphuric acid as stationary phase in order to suppress the ionisation of the acids, thereby decreasing the amount of "tailing" and obtaining better separations. The amount of the individual acids are usually determined by titration with sodium hydroxide. Moyle et al. (37) separated the lower fatty acids on silica gel with chloroform-butanol, using a phosphate buffer system as stationary phase. Gas-phase chromatography (38) is however much used for analysis of mixtures of fatty acids (18d).

The resolving power of the silica gel system decreases with increasing water solubility of the solutes. Therefore it is necessary to make the developing solvent progressively more polar in order to resolve an organic acid mixture of the complexity usually found in plant or animal tissue (36,39,40). Donaldson et al. (41,42) reported a simple device for supplying to the column a solvent which automatically and gradually increased in polarity. Good separation for many acids was

achieved, but succinic and lactic acids emerged together.

Zbinovsky and Burris (43) devised a method for the successful addition of the organic acids or their sodium salts to the column in the aqueous phase, thereby eliminating the tedious and often non-quantitative transfer of the sample from the aqueous to the non-aqueous phase.

Kinnory et al. stated in a recent report (44) that the use of benzene and ethyl ether as a solvent system gave a good separation and sharp peaks for elution of a mixture of organic acids from a silica gel column. They found that this solvent has several advantages over butanol or amyl alcohol and chloroform, since it prevents esterification of the carboxylic acids. It also separates succinic and lactic acids, which usually emerge together.

Paper chromatography has become an important method for separation and identification of both non-volatile and volatile organic acids. If the paper is irrigated with a neutral solvent, "tailing" of the spots is liable to occur. This is due to the fact that the ionised and unionised forms of the acid travel at slightly different rates. This can be avoided by addition to the solvent of either an acid to suppress the ionisation or a base to ensure complete ionisation. Volatile acids are chromatographed in the anion state, as they otherwise would evaporate off the paper.

Lugg and Overell (45,46) resolved mixtures of the non-volatile acids common in plants by sheet partition chromatography using solvents composed of n-butanol, water and formic or acetic acid.

As small amounts of inorganic salts cause tailing of the spots, it is important to have an extract free from cations. Bryant and Overell (47,48) obtained an extract free from interfering impurities by treatment with ion-exchange resins. They were then able to adapt their method to quantitative estimations by comparing the weights of the paper containing spots of the separated acids with spots from known concentration of test acids.

Hanes and Isherwood (49) achieved clean separation of mixtures of organic acids by irrigation with n-propanol containing strong ammonia. The chromatograms were dried and the spots of ammonium salts detected by spraying with a suitable pH-indicator. Similar methods have been used successfully for the separation of volatile and non-volatile acids (50,51) and different solvents have been proposed (52).

Reid and Lederer (53) devised a technique for the quantitative determination of fatty acids separated as the ammonium salts by utilising the relationship between the spot area and the logarithm of the acid content.

Isherwood and Hanes (54) carried out quantitative

estimations of the ammonium salts of a wide variety of acids by paper chromatography. The method is based upon the fact that under suitable conditions the colour of a selected indicator (Thymol blue) is unaffected by the presence of ammonium ions. The ammonium salts behave therefore as free acids, and the colour of the indicator is reduced in proportion to their concentration. The amounts of acids can then be measured colorimetrically.

The ion-exchange techniques for separation of organic acids depend upon the fact that the anions combine with positively charged groups on a synthetic resin and remain fixed to it until eluted by an aqueous solution of an acid or a base. In the latter case the acids are recovered as a solution of the salts.

Anion exchangers are classified as weak base and strong base types (55,56). The strong base type is generally the more efficient in the adsorption of acids. The capacity is higher and almost independent of the pH of the solution. Care is required however in the separation of acids on strongly basic resins when the extracts also contain sugars. Hulme (57) and Phillips and Pollard (58) reported the breakdown of sugars with the formation of lactic and glycollic acids when sugar solutions were passed through the strongly basic resin "Amberlite IRA-400" and "Dowex 2", generated in the hydroxyl form.

This breakdown does not occur if the resins are generated in the carbonate form. The capacity in this form however is lower than the capacity of the same resin in the hydroxyl form.

Schenker and Rieman (59) separated malic, tartaric and citric acids from fruit by using a strongly basic resin, "Dowex 1", in the nitrate form. Each of the acids was eluted separately by a boric acid buffer system containing sodium nitrate in increasing concentration.

Bryant and Overell (47) adsorbed the acids on the strongly basic resin "Amberlite IRA-400" which was generated in the carbonate form. The acids were eluted with a solution of sodium carbonate, the excess of which was removed by addition of the appropriate quantity of a cation exchange resin, generated in the hydrogen form. This treatment gives a cation-free extract which is suitable for analysis by partition chromatography.

However the technique of Busch et al. (60) appeared to be especially well adapted for quantitative determination of the various acids in the Krebs cycle. A strongly basic resin "Dowex 1", was used in its formate form for the adsorption of the acids. The acids were eluted with formic acid which was gradually increased in concentration by automatic means. Fractions were collected in test tubes and the formic acid was removed by evaporation. The remaining non-volatile acids were

titrated with alkali or estimated by other means. Slightly volatile acids, such as lactic acid, and acids liable to decompose during the evaporation process, could be estimated by chemical methods in the fractions before evaporation of the formic acid.

Davies and Hughes (11) and Hulme and Richardson (12), working on extracts of dried grass, removed the amino acids and cations by passing the extract through a cation exchange resin. After being adsorbed on an anion exchanger, the acids were displaced with hydrochloric acid. A partial fractionation of quinic, succinic, malic, malonic and citric was achieved, but there was some overlapping which made a quantitative estimation difficult.

None of the methods outlined above can be used alone for the complete separation of all the organic acids which occur in grass or silage. In the present work it was therefore necessary in the first instance to identify the acids in grass, and develop reliable methods for their separation and quantitative estimation. The aim was then to apply the methods to the study of these acids and their fate during wilting and silage fermentation.

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EXPERIMENTAL

EXPERIMENTAL

Various methods for separation of organic acids were investigated. These are described under the headings I, II....

I. Paper chromatography of non-volatile organic acids.

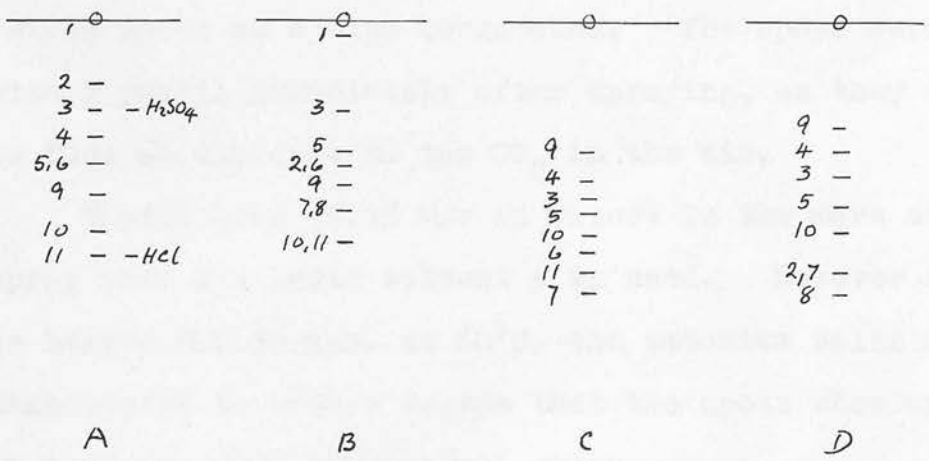
Chromatography on Whatman No.1 paper has been used in the present work for preliminary identification of the non-volatile organic acids. The following solvents were used.

A.	n-Propanol-conc. ammonia	60/40 (v/v)	(54)
B.	Phenol-water-90% formic acid	3g./1ml./1%	(61)
C.	n-Butanol-water-90% formic acid	100/85/16.8 (v/v)	(46)
D.	n-Butanol-water-glacial acetic acid	60/20/20 (v/v)	

Since A, B and D are single phase solvents and therefore unaffected by small temperature changes, they were used in tanks kept in the laboratory. Solvent C is a two phase system and was equilibrated at 21°C. for 2 days before it was used in a tank maintained at this temperature.

R_F - values are variable and considered to be unreliable. Therefore control mixtures of known acids were always run on the same sheet of paper as the unknown acids for identification purposes. Good separations of any mixture of the Krebs cycle acids could be obtained by running the papers in the appropriate solvent for 24 hours without previous equilibration in the

atmosphere. The order of separation of the acids in the above solvents was as follows:



1. Oxalic acid
2. trans-Aconitic acid
3. Citric acid
4. Tartaric acid
5. L-Malic acid
6. Malonic acid
7. Succinic acid
8. Fumaric acid
9. Quinic acid
10. Pyrrolidone carboxylic acid
11. Lactic acid

The acids in solvents B and C were removed by heating for 2 hours (D, 4 hours) at 60°C. or by leaving the paper overnight at room temperature.

Bromocresol green (400 mg. in 1 l. 95% alcohol) has been generally adopted as a spraying agent. The indicator was converted to its sodium salt by addition of 1 N-NaOH and a deep green-blue colour was obtained. The acids show up as yellow spots on a blue background. The spots were marked with a pencil immediately after spraying, as they are liable to fade on exposure to the CO_2 in the air.

Thymol blue (0.1% w/v in water) is the more sensitive spray when the basic solvent A is used. However if the paper is heated for 30 min. at 60°C. the ammonium salts are dissociated to such a degree that the spots show up clearly on spraying with bromocresol green.

II. Chromatography on silica gel.

A non-adsorbent silica gel was prepared from water glass by precipitation with 10N-HCl according to Isherwood (36). The gel was passed through a brass gauze sieve (90 mesh) and stored in an airtight container.

A modification of Isherwood's method (36,40,41,62) was first tried for the separation of the organic acids as follows:

Preparation of the column.

Silica gel (3.0 g.) and 0.5N-H_2SO_4 (3.0 ml.) were thoroughly mixed and to this was added chloroform (equilibrated against

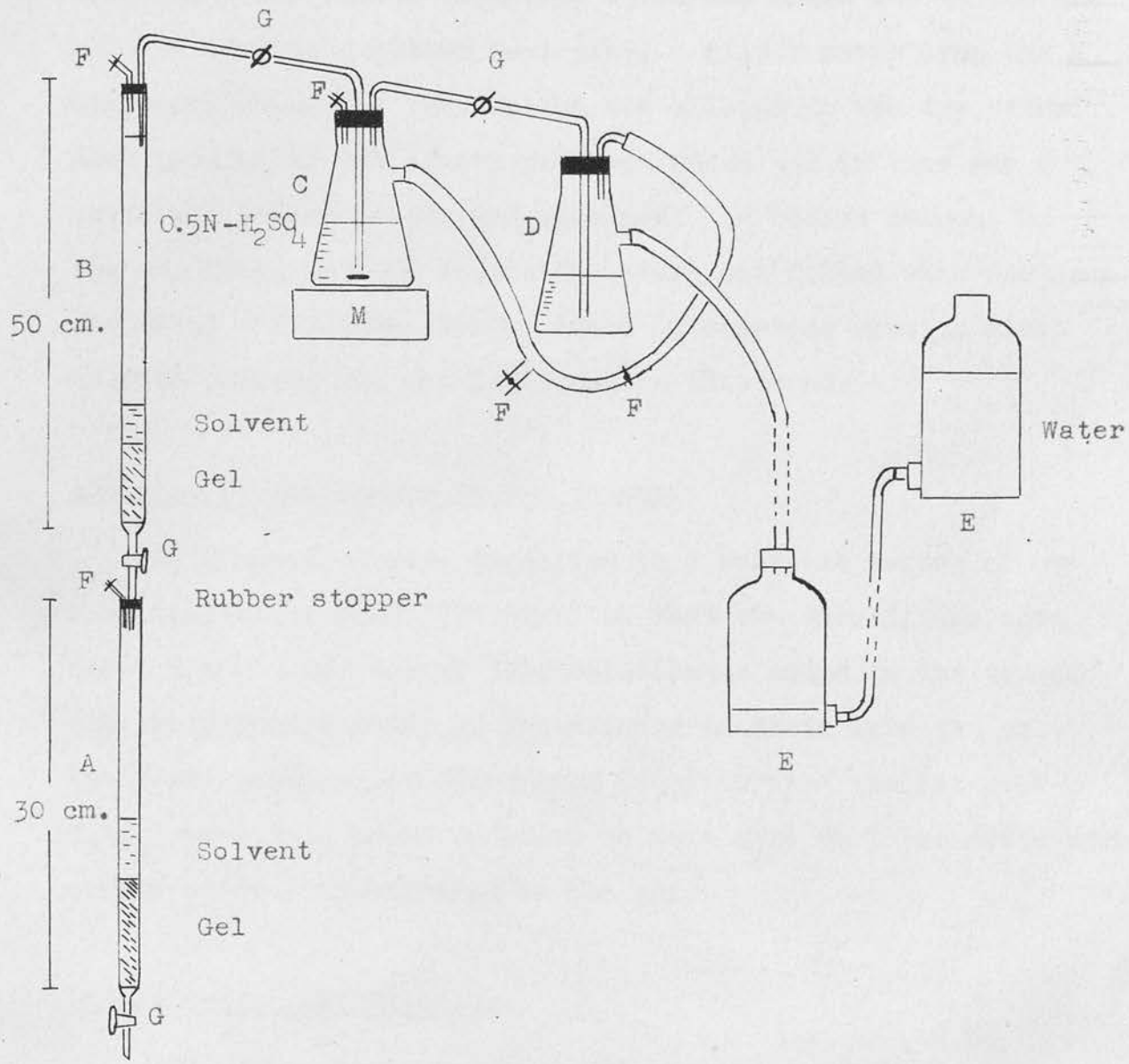


Fig. 2. GRADIENT ELUTION APPARATUS

v. pp. 31, 32, 33.

0.5N-H_2SO_4) until a thin slurry was obtained. This was then poured in small portions into a chromatography tube (30 cm. long and 1 cm. inside diameter) which was drawn out at the end and fitted with a cotton wool plug, a filter paper disc and a stopcock.(Fig.2A). The column was allowed to run dry after each portion of the slurry had been added and in this way a uniformly packed column was obtained. A second column, B, (50 cm. long, 1.3 cm. inside diameter) was filled with the same amount of silica gel slurry, added in only one portion since uniform packing was not important in this case.

Addition of the sample to the column.

Acid mixtures were dissolved in a suitable volume of n-butanol-chloroform (50-50v/v) so that the normalities were about 0.05. One ml. of this solution was added to the top of the analytical column, A, and allowed to drain into the gel. Two 1 ml. portions of chloroform (equilibrated against 0.5N-H_2SO_4) were then added in order to make sure that the acids were quantitatively transferred to the gel.

Determination of the acids.

After the sample had been transferred to the gel, the solvent (n-butanol-chloroform) was passed through the column

at a rate of about 15 ml./hour. The eluate was collected in 2 ml. fractions in test tubes by means of a mechanical fraction collector. The acid content of each tube was determined by titration with 0.01N-NaOH (CO_2-free), standardised with potassium hydrophthalate. The following indicator solution was used: 2 ml. of 1% phenolphthalein in alcohol were diluted to 250 ml. with abs. alcohol. 1 ml. of this solution was added to each test tube and CO_2-free air was bubbled through the solution for 30 sec. before starting the titration. 0.01N-NaOH was then added from a 5 ml. micro-burette to neutrality. The amount of acid was calculated after the formula:

..... V · E · N = mg. acid .....

V = volume of NaOH

E = equivalent weight of acid

N = normality of NaOH.

Experiment 1. Determination of the chromatographic properties of the gel.

Oxalic acid, which is a strong acid, is adsorbed to a certain extent on untreated silica gel, and on HCl treated gel if water is used as stationary phase. This would give a noticeable tail, or an unsymmetrical titration curve. If oxalic acid can be

ELUTION OF OXALIC ACID FROM A SILICA GEL COLUMN

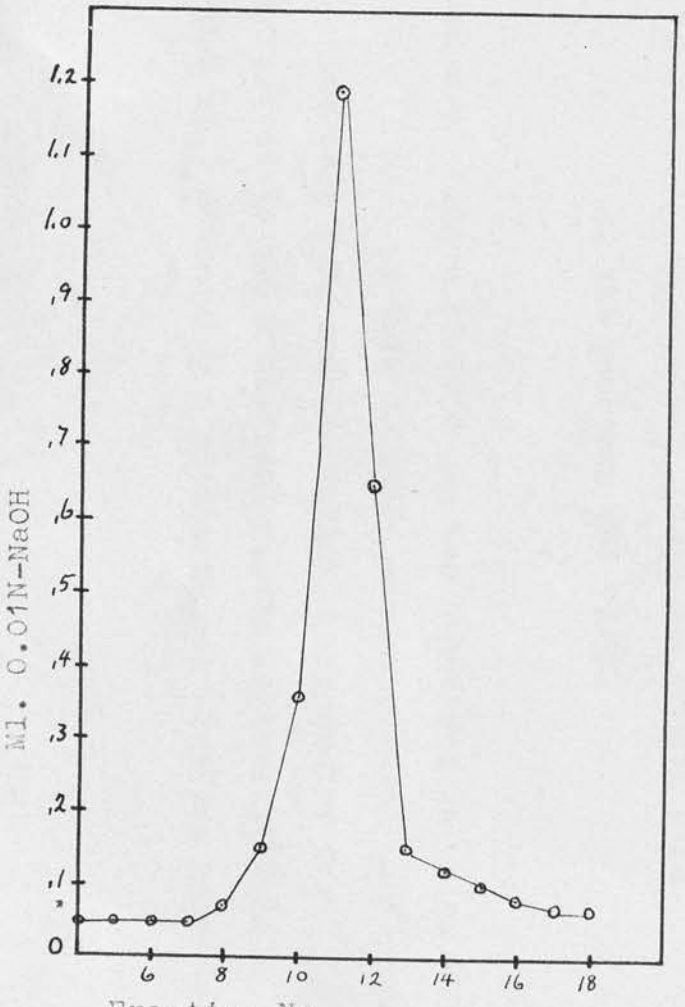


Fig.3

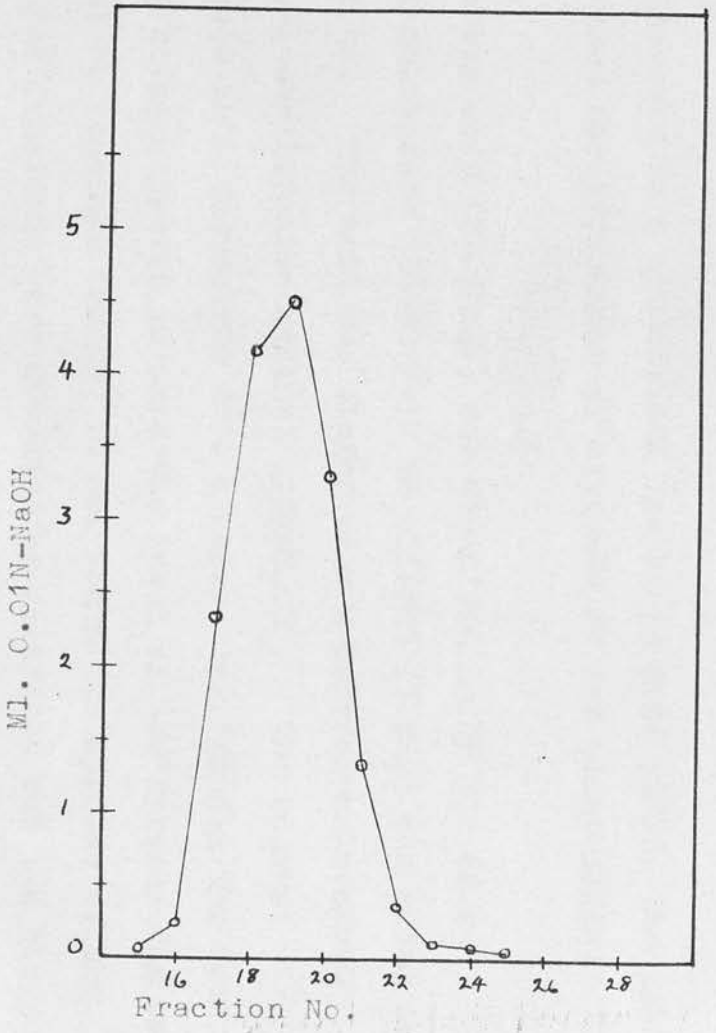


Fig.4

eluted, however, in a symmetrical "peak" in good yield, the gel can be used for separation of mixtures of the plant acids.

a. Oxalic acid (77.2 mg.) was dissolved in 50 ml. of n-butanol-chloroform (50-50v/v). An aliquot (1 ml.) was added to the column. The acid was eluted with n-butanol-chloroform (35-65v/v), equilibrated against 0.5N-H_2SO_4. The solvent was introduced into the column from a separating funnel, the rate of flow being adjusted so that the level of the solvent was as constant as possible and ca. 10 cm. above the surface of the gel. The fractions were titrated as described and the titres plotted graphically. A curve of sufficient symmetry was obtained. (Fig.3).

The total acid eluted ≡ 2.39 ml. 0.0100N-NaOH

≡ 1.51 mg. oxalic acid

i.e. the recovery was 97.5%.

b. Ammonium oxalate (0.2950 g.) was dissolved in water (50 ml.). An aliquot (2 ml.) of this solution was evaporated to dryness in a desiccator over P_2O_5 and the following procedure for transferring it to the analytical column followed.(40,62): Water (0.2 ml.) containing a little thymol blue was added and a

concentrated solution of the salt obtained. Silica gel (0.5 g.) was then added and the mixture stirred with a thin glass rod. A thick cream was obtained by addition of dry n-butanol-chloroform (5-95v/v). 6N-H_2SO_4 was added until the indicator changed to pink (less than 1 drop required). More solvent was added and the slurry transferred to the analytical column. The beaker was rinsed with two 5 ml. portions of solvent. A symmetrical curve (Fig.4) was obtained upon elution with n-butanol-chloroform (35-65v/v).

The total acid eluted ≡ 15.96 ml. 0.0100N-NaOH

≡ 11.33 mg. ammonium oxalate

i.e. the recovery was 96.0%.

Experiment 2. Separation of prepared mixture of succinic, malic and citric acids.

A solution was prepared containing succinic acid (0.2950 g.), malic acid (0.3250 g.) and citric acid (0.2380 g.) in 100 ml. n-butanol-chloroform (50-50v/v). 1 ml. of this solution was added to the top of the analytical column (Fig.2A), followed by 2 portions (2 ml.) of the developing solvent, n-butanol-chloroform (5-95v/v), to wash the material from the sides of the tube. The tube was filled with solvent to a height of 10 cm.

above the surface of the gel and elution started. The concentration of n-butanol in the solvent was gradually increased from 5% to 35% by a gradient elution technique as shown in fig.2:

A = the analytical column.

B = equilibrium column (3 g. silica gel, 2-3 cm. solvent and ca. 30 cm. 0.5N-H_2SO_4).

The reason for introducing column B was to ensure complete equilibration between the solvent and the stationary phase (62).

C = mixing flask (250 ml. filter flask) containing 200 ml. of n-butanol-chloroform (5-95v/v), equilibrated against 0.5N-H_2SO_4.

D = reservoir containing 200 ml. of n-butanol-chloroform (50-50v/v), not equilibrated.

E = adjustable water reservoirs (1 l.).

M = magnetic stirrer.

The two columns were supplied with an air-inlet, so that they could be connected by means of a rubber stopper without altering the pressure inside the column and thus disturbing the surface of the silica gel. The elution was started by opening all the stopcocks G and closing all the screwclips F. The

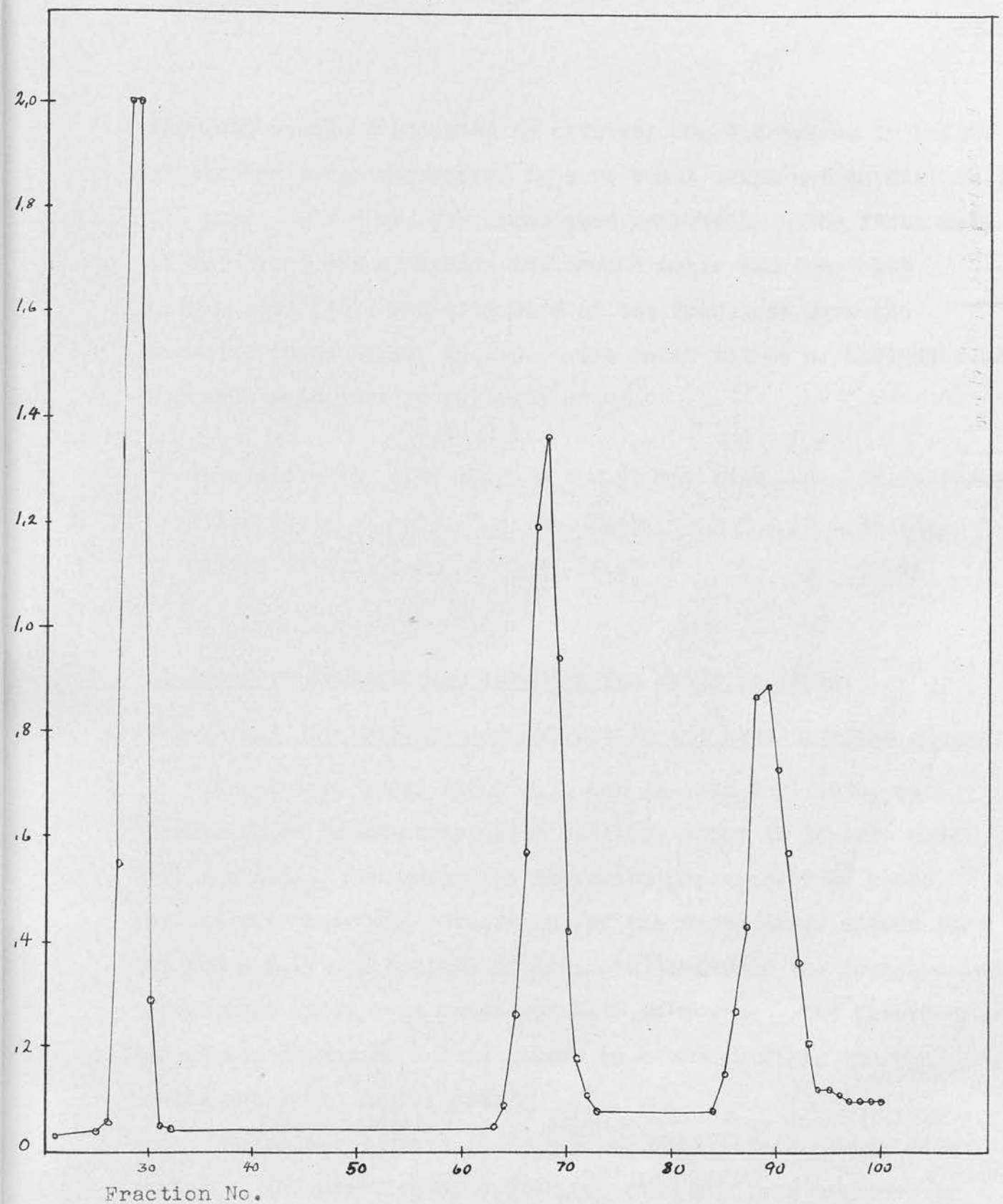


Fig.5 SEPARATION OF ORGANIC ACIDS ON SILICA GEL

flowrate could be adjusted by altering the difference in height of the two water reservoirs E, care being taken not to disturb the gel. 2 - 3 ml. fractions were collected. The first acid to be eluted was succinic, the second malic and the third citric acid (36), and titration of the fractions gave the expected three peaks, fig. 5. The total titres of 0.0103N-NaOH for each acid were as follows:

Succinic acid,	4.81 ml.	≡	2.91 mg.	acid	≡	98.7%	recovery
Malic acid,	4.54 "	≡	3.14 "	"	≡	96.7%	"
Citric acid,	3.43 "	≡	2.27 "	"	≡	95.0%	"

Attempts to separate and identify the acids in grass.

Experiment 3. Preliminary extraction and investigation of grass.

Fresh lawn grass (50.2 g.), cut on 19th May, 1954, was freeze-dried immediately after cutting, using an Edwards Model 111 machine. The weight of the dried grass after 20 hours drying was 12.10 g. Ovendrying of the fresh grass showed that it had a moisture content of 80%, and therefore the freeze-dried grass must still have contained 4.1% moisture. The freeze-dried grass was ground to a fine powder in a hammer mill, and the acids extracted as follows:

The powdered grass (9.20 g.) was made into a slurry with water. The addition of 0.2N-H_2SO_4 (100 ml.) was required to

bring the pH down to 2. The mixture was then centrifuged and the supernatant liquid decanted. The residue was washed twice with 20 ml. portions of water. The washings were combined with the acid extract and the solution filtered to remove some remaining floating solid material. The solution was then neutralised with 2N-NaOH, using phenolphthalein and concentrated under reduced pressure (bath temp. 40-50°C.). 2N-H_2SO_4 was added to pH=2 (indicator paper) and the solution was transferred to a measuring cylinder and made up to 18 ml. with water. A precipitate had been formed during the concentration. After mixing by stirring with a glass rod, the extract was allowed to stand until the precipitate settled. The organic acids were then transferred to the non-aqueous phase according to Isherwood (36):

10 ml. of the clear supernatant solution were mixed with silica gel (12 g.) and the mixture thoroughly stirred and pressed against the side of the beaker with a glass rod until a superficially dry powder was obtained. About 50 ml. of n-butanol-chloroform (50-50v/v) were added and the mixture carefully transferred to a glass tube (length 20 cm., diameter 5.5 cm.), fitted with a perforated disc and filter paper at the end. The column was allowed to run dry, refilled with solvent, and 350 ml. of the filtrate were collected. A further 50 ml. were collected separately and titrated with 0.1-NaOH (phenolphthalein).

0.25 ml. were required and the extraction was complete. The main bulk of the filtrate was then transferred to a separating funnel and titrated with 0.1N-NaOH with frequent shaking. 23 ml. 0.1N-NaOH were required. The lower solvent layer was separated and washed twice with 15 ml. portions of water. The washings were added to the aqueous layer and the solution concentrated *in vacuo* to about 2 ml. (bath temp. 40-50°C.), transferred to a small beaker and dried *in vacuo* over P_2O_5. The residue was dissolved in 4N-H_2SO_4 (0.7 ml.) and the solution mixed with silica gel (1.0 g.). 10 ml. of tert.-amyl alcohol-chloroform (50-50v/v) were added and the mixture quantitatively transferred to a glass tube (length 25 cm., diameter 1.3 cm., similar to A, fig. 2) which was allowed to drain, and the eluate collected. The beaker was rinsed with some of the eluate and finally with 5 ml. of the solvent. When this had been absorbed, the tube was refilled with solvent and 30 ml. eluate collected. Titration of a further 5 ml. eluate showed that the extraction was complete.

4.5 ml. 0.01N-NaOH were required to neutralise 1 ml. of the amyl alcohol-chloroform extract (not corrected for acidity of the solvent).

An aliquot (2 ml.) of this extract was added to the analytical column A (fig.2) and the elution carried out as described under experiment 2. 120 fractions of about 2 ml. each were collected.

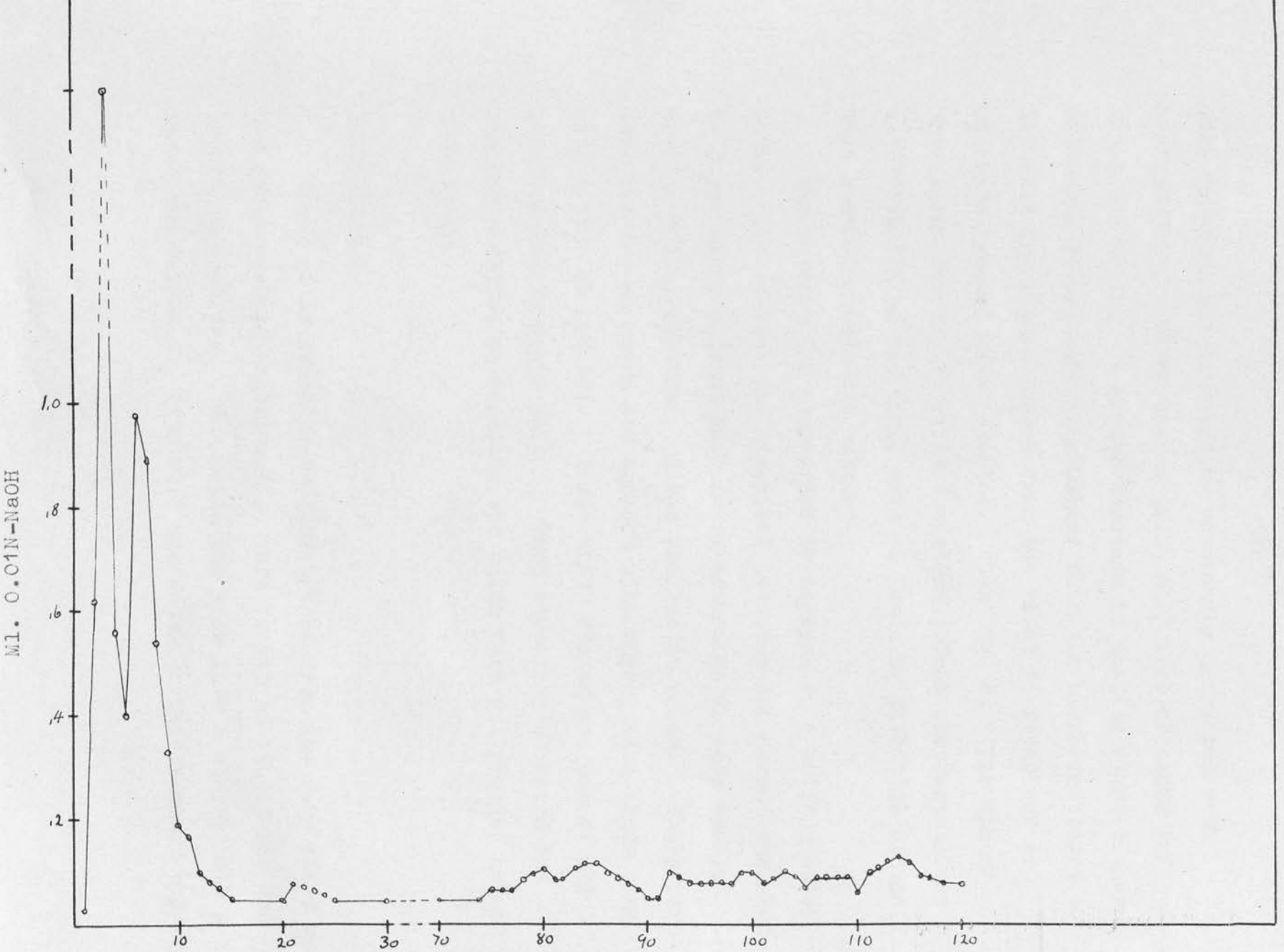


Fig.6. ATTEMPTED SEPARATION OF ACIDS FROM GRASS ON SILICA GEL

Each fraction was titrated as previously described with 0.0103N-NaOH. Blank titres (0.04 ml.) were obtained for fractions 16-74. A slight increase in acidity started thereafter, and titres generally higher than the blank continued to the last fraction. Apart from the first 15 fractions no definite "peaks" were detected. See fig. 6. The sum of the titres for the 15 first fractions (blank subtracted) was 6.86 ml., 76% of the total acid as found by titration of the amyl alcohol-chloroform extract.

The extract was evaporated to dryness on a boiling water bath. The residue was dissolved in water and chromatographed on paper using solvent A. As reference acids were spotted citric, malic, succinic, quinic and lactic acids. The papers were run for 20 hours and sprayed with thymol blue (made blue with 1 drop of N-NaOH). Three spots showed up, one of which corresponded to malic acid. Some streaking occurred and further attempts to identify the acids were not carried out at this stage.

Experiment 4.

104.7 g. of fresh grass (cut 1/6/54 from the same place as the grass used in experiment 3) were frozen in liquid air and ground in a mortar. The solid was made into a slurry with 1 l. of water and 0.2N-H_2SO_4 (150 ml.) was added. This reduced the pH

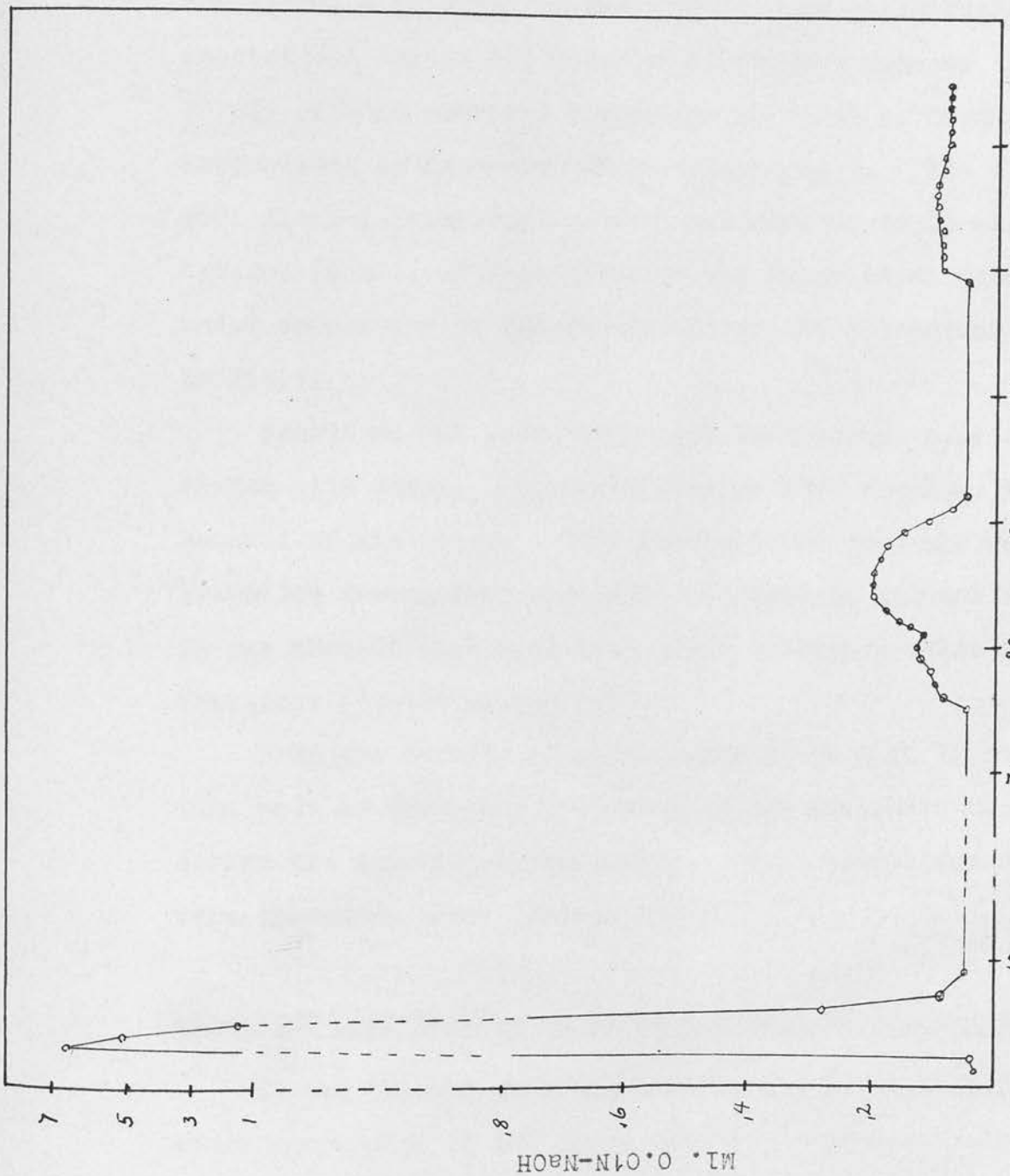


Fig.7 ATTEMPTED SEPARATION OF ACIDS FROM GRASS ON SILICA GEL

to 2.1. The mixture was left for 2 hours with occasional stirring, filtered through a plug of glass wool and washed with water. The combined filtrate (about 2.5 l.) was concentrated in vacuo to about 30 ml. and centrifuged. The supernatant liquid and water washings were made up to 50 ml. 10 ml. of this extract, equivalent to 20.95 g. fresh grass, were treated as described under experiment 3. The final amyl alcohol-chloroform extract was made up to 26 ml. An aliquot (2 ml.) of this solution was analysed as described under experiment 3, and the result of the titrations is shown in fig.7.

Fractions 3-7 were evaporated to dryness, acidified and shaken with ether. A crystalline residue remained after removal of the ether. This proved to be only one acid which travelled faster than succinic acid both in solvent A and C. It was assumed that fractions 75-93 contained malic acid and fractions 110-120 citric acid.

From the results of experiments 3 and 4 it is obvious that most of the acids present in grass must have been lost during the extraction procedure. Other extraction methods were therefore investigated.

Attempted purification of grass extracts by electrodialysis.

It was thought that the acids could be separated from the other components of the grass extract by electrodialysis.

Experiment 5.

A standard mixture containing succinic acid (150 mg.), malic acid (170 mg.) and citric acid (160 mg.) in 50 ml. water was prepared. Unless otherwise stated the titrations in the following experiments were carried out using phenolphthalein as indicator.

Titration of 1 ml. of the standard mixture required 13.90 ml. 0.0108N-NaOH (calc. 13.88 ml.). The apparatus used for electrodialysis was similar to that described by Macpherson (63). A diagram of the cell, which is made from "Perspex" sections is shown in fig.8.

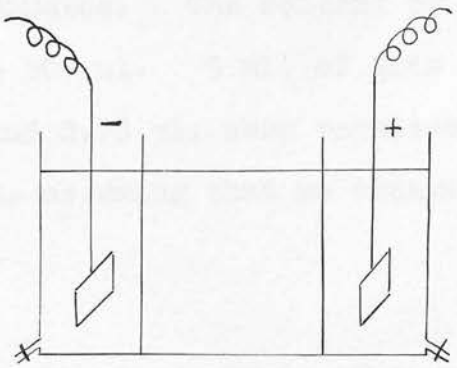


Fig. 8

The end compartments, of about 100 ml. capacity, were fitted with draining tubes. The capacity of the centre compartment was about 150 ml. The cathode membrane was vegetable parchment and the anode membrane formolised gelatin

on a cloth base. The electrodes (about 10 sq.cm.) were made of thin Pt-foil. A current of about 1 amp. was employed in the following experiment at a potential of about 200 v. The contents of the compartments were cooled and the solution in the centre one continuously stirred.

20 ml. of the standard acid solution were placed in the centre compartment and diluted with water to about 150 ml. The end compartments were filled up with water and the current switched on. NH_4OH was added continuously to the centre compartment to neutral reaction (indicator paper) for the first 20 minutes after which the solution remained neutral without any further addition. The electrodialysis was continued for a further 25 minutes. The content of the anode compartment was made up to 500 ml. 5 ml. of this were titrated with 0.0108N-NaOH and 2.78 ml. were required. Thus 100% of the acids were recovered, assuming that no changes had taken place in the acids.

Experiment 6. Electrodialysis of a grass extract.

130.8 g. of fresh lawn grass (cut 15.6.54) were dried at 95-100°C. to constant weight. Moisture 86.0%. The dried grass (18.3 g.) was boiled gently for 20 min. with 500 ml. of water (11) and filtered through a glass wool plug. The residue was washed with 100 ml. of water. This procedure was repeated

once and the grass was pressed against the sides of the funnel. The extract (pH=6.20) was concentrated under reduced pressure to about 100 ml., transferred quantitatively to the centre compartment of the cell and electrodialysed as described under experiment 5. The current exceeded 1 amp. after a few minutes and the ammeter (max. reading 1 amp.) had to be disconnected. There was a considerable evolution of chlorine from the anode compartment. To keep the temperature as low as possible, the anode compartment was emptied frequently and refilled with water. The acid mixture in the anode compartment contained much coloured matter. It was neutralised with N-NaOH (23.0 ml.), concentrated and made up to 25 ml. A sample (20 ml.) was evaporated to dryness on a steam bath, aided by a gentle current of air. The residue was dissolved in ca. 1 ml. of 6N-H_2SO_4 and mixed with 1.5 g. of silica gel. The mixture was completely transferred to a glass tube (see experiment 3) and the acids eluted with tert.-amyl alcohol-chloroform (50-50v/v), 50 ml. eluate being collected. This was evaporated to dryness and made up to 10 ml. with tert.-amyl alcohol-chloroform (50-50v/v) in order to obtain a suitable amount of acids for analysis. 2 ml. were added to the analytical column and the separation carried out as described under experiment 2. 110 fractions were collected and every second fraction titrated in order to locate the acids. The contents of the other acid containing

fractions were evaporated to dryness on a water bath, dissolved in a little water and examined by paper chromatography using solvents A and C.

Fractions 3-5 gave one spot in both solvents corresponding to the acid mentioned in experiment 4(fr. 3-7).

Fractions 29-31 gave one spot travelling at the same rate as succinic acid in both solvents.

Fractions 37-41 gave 2 spots in solvent A and 1 spot in solvent C (streaking). The spot in solvent C and 1 spot in solvent A corresponded to that of pyrrolidone carboxylic acid. The other spot in solvent A corresponded to that of malonic acid.

Fractions 71-79 gave 1 spot corresponding to that of malic acid in both solvents.

Fractions 90-100 gave 2 spots in both solvents. One spot corresponded to citric acid while the other spot was found between citric and malic acid in solvent A and remaining on the starting line in solvent C.

As test acids were employed succinic, malic, citric, tartaric, malonic, glutaric, quinic, pyrrolidone carboxylic, α-ketoglutaric, trans-aconitic and lactic acids.

It was thus concluded that the analysed grass contained

succinic, pyrrolidone carboxylic, malic and citric acids, possibly malonic acid and at least three other acids.

Experiment 7.

100.0 g. of fresh lawn grass (cut 28/7/54, moisture 80%) were soaked in boiling methylated spirit (750 ml.). After filtration through a glass wool plug, the grass was extracted with boiling water as described in the previous experiment. The combined alcohol and water extracts were concentrated under reduced pressure and made up to 100 ml. This extract was very dark in colour. To an aliquot (50 ml.) was added alcohol (150 ml.). A coloured precipitate was formed which was removed by filtration and washed with aqueous alcohol. The filtrate and washings were concentrated to 100 ml. and used for two experiments.

(i) Purification by electrodialysis. An aliquot (25 ml. ≡ 2.5g. dry wt. of grass) was electrodialysed. Titration of the solution in the anode compartment required 21.8 ml. of 0.1N-NaOH. The salt solution was concentrated under reduced pressure, transferred to a small beaker and evaporated to dryness over P_2O_5. The sample was transferred to the analytical column as described under experiment 1(b), and the elution carried out in the usual way. It was noted that a transparent band started

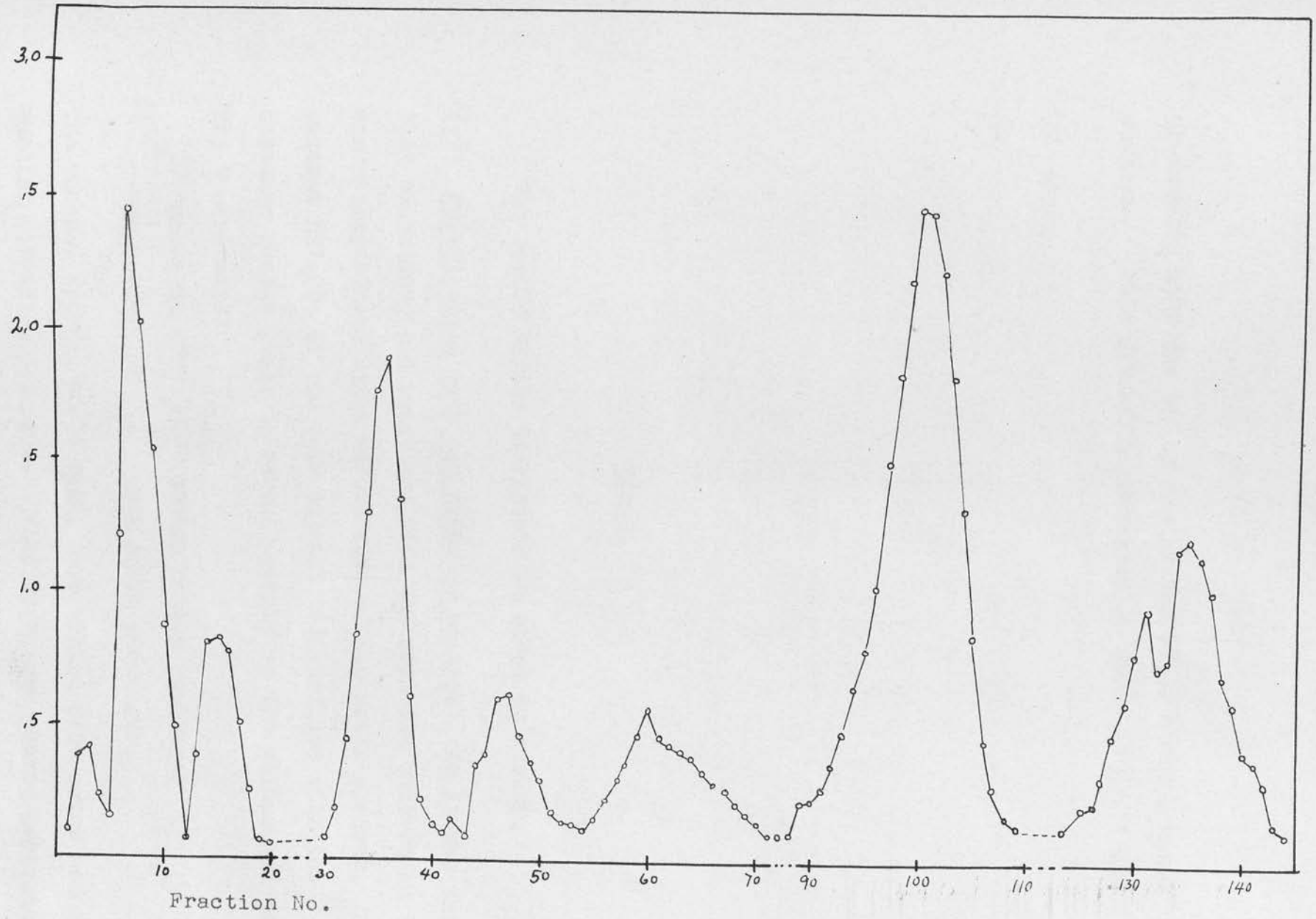


Fig.10. SEPARATION OF ACIDS ON SILICA GEL AFTER ELECTRODIALYSIS OF THE EXTRACT

to develop near the top of the column after about 4 hours elution. This gradually increased in width to ca. 1 cm. (Fig.9).

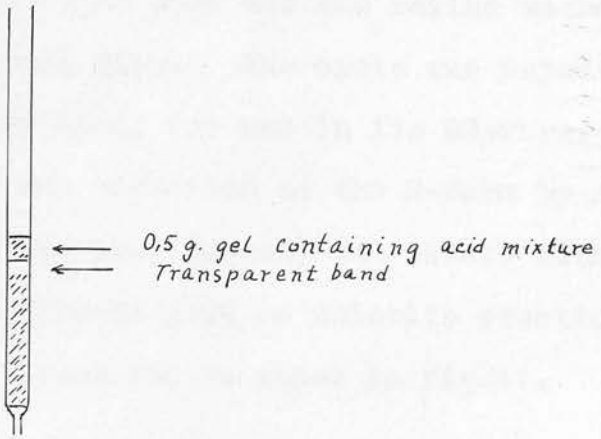


Fig.9.

The result of the titrations is shown in fig.10.

(ii) Purification with ion exchange resins. As it was thought that oxidation, and reactions with the chlorine evolved in the anode compartment might affect some of the acids present, another aliquot of the same extract was purified with ion exchange resins prior to chromatography on the silica gel column. The resins were:

"Zeo-Karb-225" - strong cation exchanger

"De-Acidite-G" - weak anion exchanger.

The resins, approx. 40-70 mesh, were washed with water and the smaller particles decanted. They were then packed separately

into two columns (length 7 cm., diameter 0.8 cm.) and irrigated with 2N-HCl, distilled water, N-NaOH, and distilled water in order to remove all soluble impurities. The effluents were tested with pH-paper between each step. An excess of ca. 2 bed volumes of HCl and NaOH were used and the resins washed to neutral reaction between each step. The cycle was repeated once. The anion exchanger was now ready for use in its OH-form.

The cation exchanger was converted to the H-form by irrigation with 2N-HCl (2 bed vol. excess) and washed with distilled water till the effluent gave no chloride reaction.

The two columns were connected as shown in fig.11.

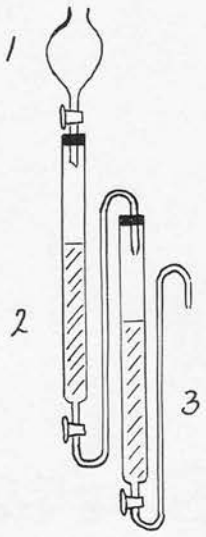


Fig.11.

1 - 50 ml. separating funnel.

2 - Cation exchange resin (H).

3 - Anion exchange resin (OH).

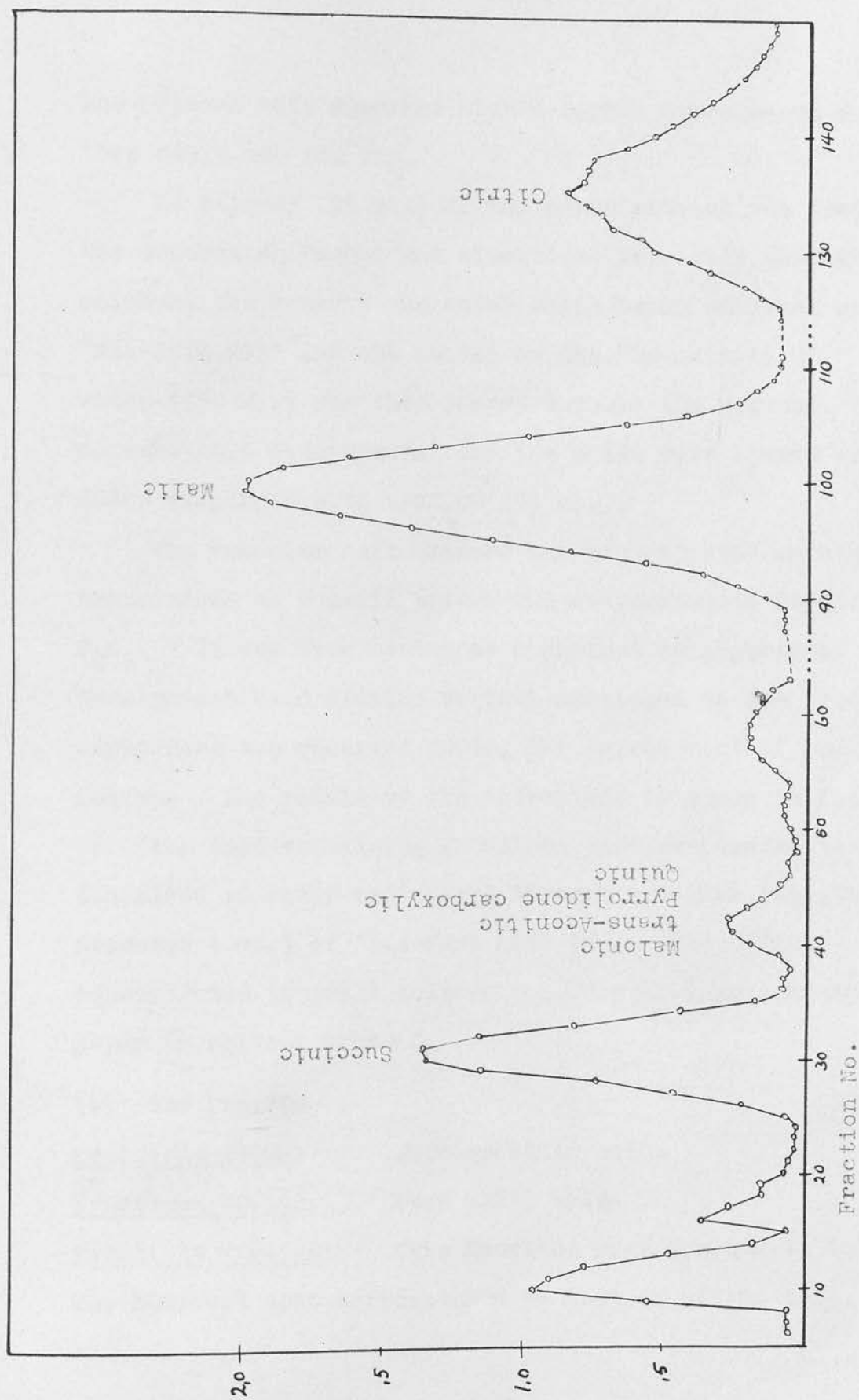


Fig.12 SEPARATION OF ACIDS ON SILICA GEL AFTER TREATMENT OF THE EXTRACT WITH ION EXCHANGE RESINS

The columns were supplied with a syphon arrangement so that they would not run dry.

An aliquot (25 ml.) of the grass extract was transferred to the separating funnel and allowed to percolate through the two columns, the cations and amino acids being adsorbed on the "Zeo-Karb 225" and the anions on the "De-Acidite G". Distilled water (150 ml.) was then passed through the columns. The columns were disconnected and the acids were eluted from the anion exchanger with N-NH_4OH (50 ml.).

The ammonium salt mixture was concentrated on a water bath, transferred to a small beaker and evaporated to dryness over P_2O_5. It was then treated as described in experiment 1(b). A transparent band similar to that mentioned in the previous experiment was observed during the development of the silica gel column. The result of the titrations is shown in fig.12.

The acid containing fractions were evaporated to dryness, dissolved in water and passed through a column (length 3 cm., diameter 1 cm.) of "Zeo-Karb 225" (H). The effluents were concentrated to small volumes (ca. 0.5 ml.) and chromatographed on paper in solvent A and C.

(i) See fig.10.

Fractions 31-39: Pure succinic acid.

Fractions 90-108: Pure malic acid.

Fractions 124-146: This fraction gave 3 spots in both solvents. The heaviest spot corresponded to that of citric acid.

(ii) See fig.12.

Fractions 8-14: One spot, corresponding to the acid mentioned in experiment 6 (fr. 3-5).

Fractions 15-20: Three spots in solvent A and only one in C. The acids were not identified.

Fractions 25-36: Pure succinic acid.

Fractions 38-46: Four spots in both solvents corresponding to malonic, trans-aconitic, quinic and pyrrolidone carboxylic acids.

Fractions 55-65: No definite spot in either solvent.

Fractions 89-111: Pure malic acid.

Fractions 125-150: Pure citric acid.

The two succinic acid fractions were combined and evaporated to dryness. The acid crystallised in a pure state with a melting point of 184°C. alone and mixed with authentic succinic acid.

The amounts of succinic, malic and citric acids found are listed in table 2.

TABLE 2. Amounts of acids found in grass.

Acid	Electrodialysis			Ion exchange resins		
	Titre(ml.) 0.0108N-NaOH	mg.acid	% dry wt.	Titre(ml.) 0.0108N-NaOH	mg.acid	% dry wt.
Succinic	8.56	5.46	0.22	7.60	4.85	0.19
Malic	20.00	14.48	0.58	17.69	12.80	0.51
Citric	9.53	6.59	0.26	8.94	6.18	0.25

Most of the coloured matter present in the grass extract was adsorbed by the "Zeo-Karb 225" resin and a little was adsorbed by the "De-Acidite G". The ammonium salt solution finally analysed on silica gel was slightly brown in colour, but did not contain as much coloured matter as the extract obtained by electrodialysis. Further experiments were carried out using ion-exchange resins for purification of the extract.

Regeneration of the resins: The Zeo-Karb 225 was irrigated with N-NaOH (50 ml.). Most of the coloured matter was eluted during this stage. The resin was washed with water (100 ml.) and converted to the H-form by irrigation with 2N-HCl (50 ml.). The resin was ready for use again after washing with distilled water (150 ml.) till a negative test for chloride was obtained.

The De-Acidite G, although in the OH-form after use, was irrigated with N-NaOH (50 ml.) and washed with distilled water till neutral reaction.

Experiment 8.

A sample of fresh perennial rye grass* (cut 12/8/54, Moisture 87.7%) was extracted with alcohol and water as described in experiment 7 and made up to 100 ml. An aliquot (10 ml.) was passed through the two ion-exchange resins and analysed on a silica gel column. Two alterations in the

* 100.0 g.

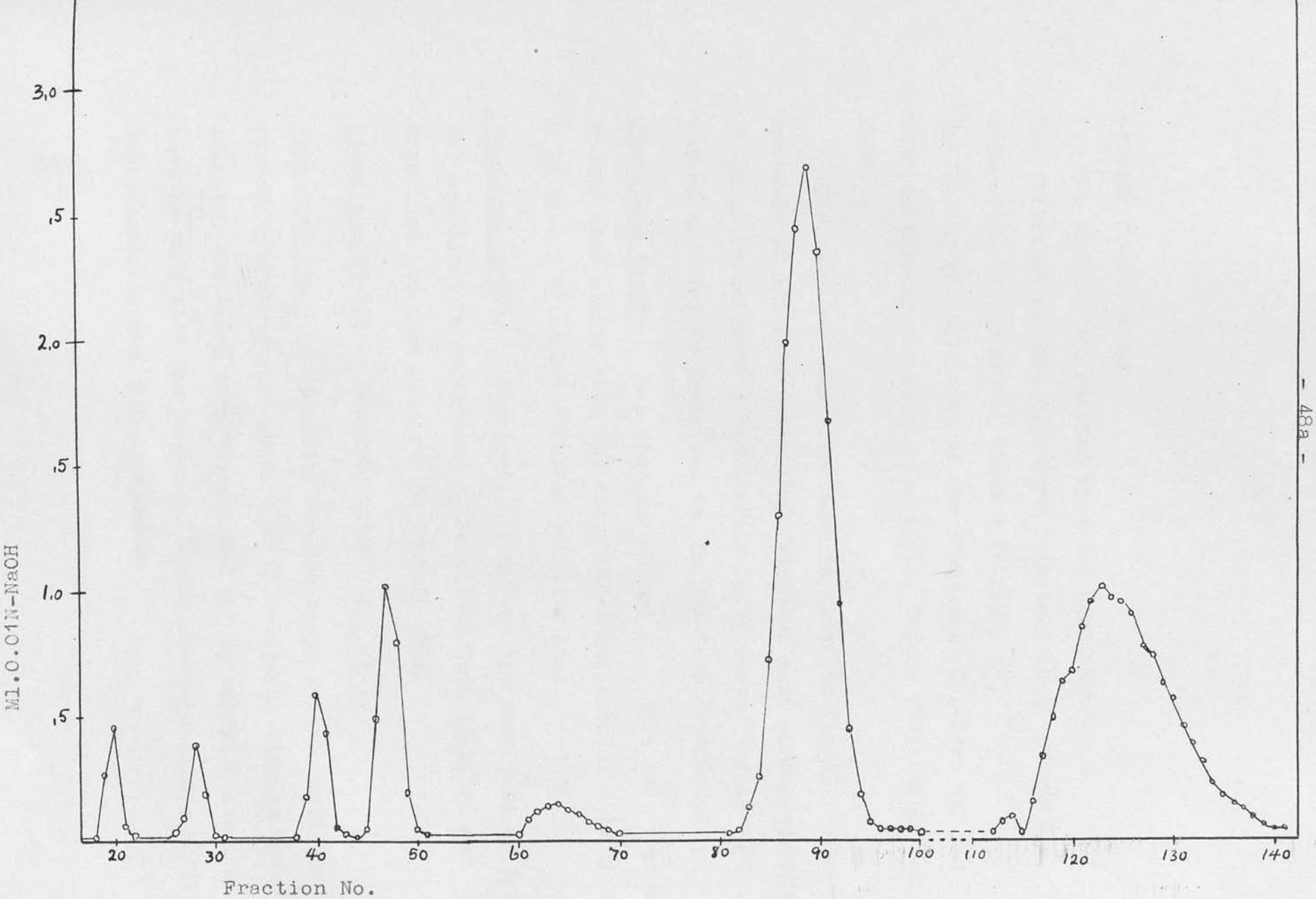


Fig.13 ACIDS FROM RYE GRASS

procedure were made:

1. The elution was started by using pure chloroform (equilibrated against 0.5N-H_2SO_4) instead of 5% n-butanol-chloroform in the mixing flask C (fig.2).

2. During the titration of the fractions, CO_2-free air was bubbled through the liquid for 1 min. before addition of the NaOH.

The result of the titrations is shown in fig.13. The contents of the acid containing fractions were chromatographed on paper in solvents A and C after being passed through the cation exchanger as described in the previous experiment.

Fractions 19-21: One spot in solvent A. This acid travelled faster than citric acid but slower than malic acid. In solvent C it gave a 1" streak from the starting line.

Fractions 27-29: One spot. This was the same acid as the one mentioned in experiment 7, fractions 8-14 (fig.12) and travelled the same distance as fumaric acid.

Fractions 38-42: Succinic acid.

Fractions 45-50: 3 spots, the two being malonic acid and pyrrolidone carboxylic acid. An unsuccessful attempt to identify the third acid was carried out by running this fraction side by side with the suspected acids, trans-aconitic, oxalic, tricarballylic and glycollic acids.

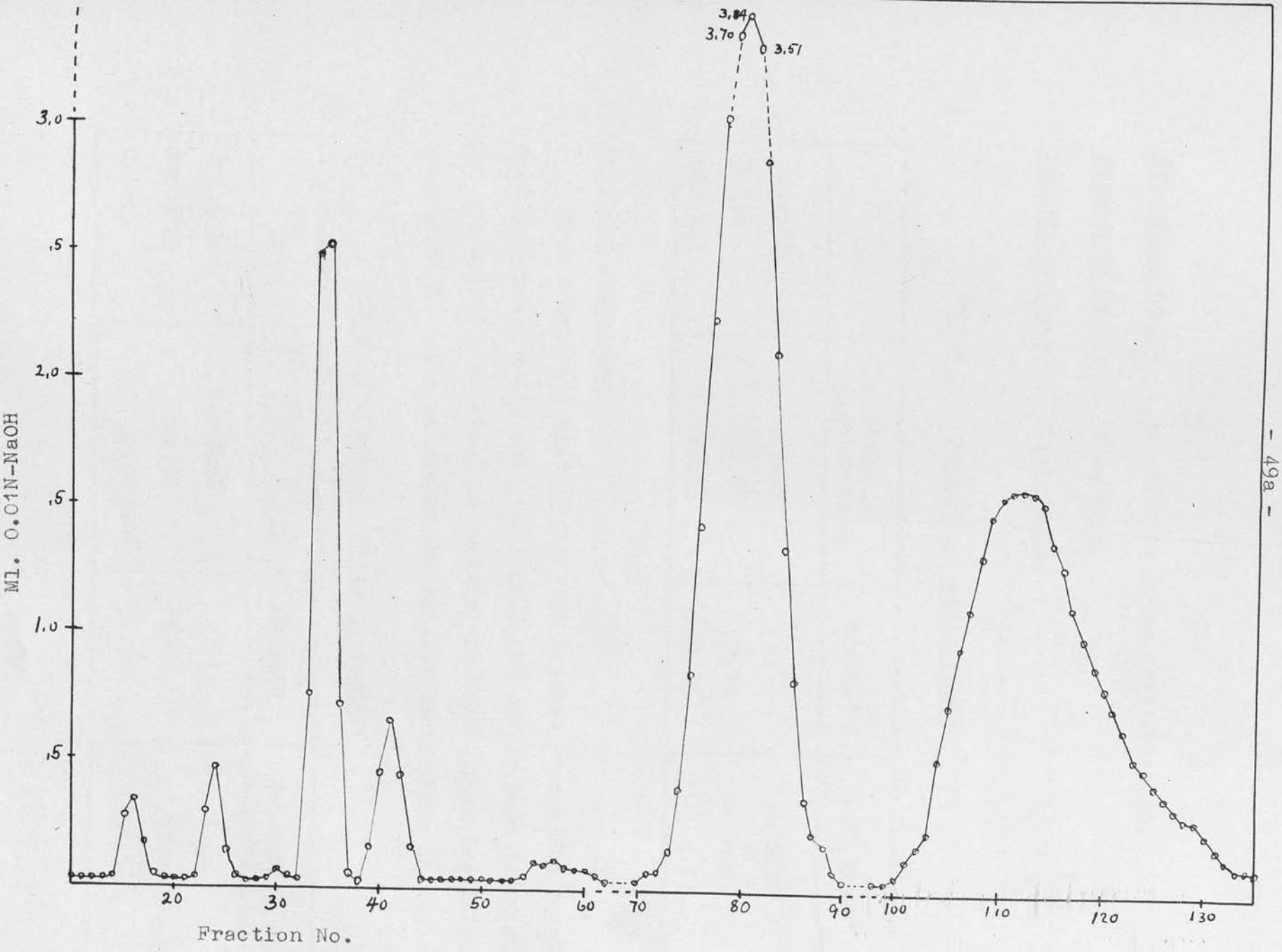


Fig.14 RECOVERY EXPERIMENT

Fractions 60-70: No definite spots were obtained.

Fractions 82-95: Malic acid.

Fractions 116-136: Citric acid.

TABLE 3. Amounts of acids found.

Acid	Titre (ml.) 0.0108N-NaOH	mg.acid	% of dry wt.
Succinic	1.23	0.78	0.06
Malic	14.83	10.74	0.87
Citric	12.56	8.69	0.71

Recovery experiment.

To a sample (10 ml.) of the same extract were added succinic acid (4.0 mg.), malic acid (10.0 mg.) and citric acid (10.0 mg.) and the solution analysed in exactly the same way as described in experiment 8. The result of the titrations is shown in fig.14.

TABLE 4. Amounts of acids found.

Acid	Titre (ml.) 0.0108N-NaOH	mg.acid	recoveries in %
Succinic	6.44	4.11	83.4
Malic	26.29	19.04	83.0
Citric	24.20	16.74	80.5

III. Chromatography on ion-exchange resin.

Materials.

Zeo-Karb 225 cation exchanger, analytical grade (40/70 mesh), nominally 4.5% cross-linked, was obtained from the Permutit Company Ltd. It was washed and generated as previously described (p.44). Usually it was irrigated with 2N-NaOH after use in order to remove as much as possible of the adsorbed coloured matter. The same material was used 5-6 times before it was discarded. By this time the colour even after regeneration was much darker than that of the fresh resin.

Amberlite IRA-400 - anion exchanger, analytical grade, was obtained from the British Drug Houses Ltd. It was ground in a mortar and passed through a 100 mesh sieve, thereafter suspended in distilled water. The cloudy supernatant fluid containing the smaller particles was decanted after 10 min. The coarser particles which had settled in that time were stirred with a fresh portion of distilled water, the excess of which was decanted. This was repeated until the wash water was clear and colourless. The resin, which was supplied in the hydroxide form, was converted to the chloride form by suspending it in 2N-HCl in a beaker with occasional stirring for several hours. It was then transferred to a chromatographic tube (length 40 cm., diameter 4 cm.) with a cotton wool pad at

the bottom, where it settled down to a 15 cm. long column. The excess HCl was removed by washing with distilled water until the pH was 7 (pH-paper). The resin was then converted to the hydroxide form again by irrigation with N-NaOH until the effluent gave negative test for chloride. The excess NaOH was washed off the column with distilled CO_2-free water and the resin was generated in the formate or acetate form by irrigation with formic acid (17%) or acetic acid (30%). Two bed-volumes of acid were allowed to percolate through the column after the acid had broken through. The resin was washed with several bed-volumes of water and stored wet in a bottle.

General procedure for separation of non-volatile organic acids on an Amberlite IRA-400 column.

The separation technique (60,64) adopted was as follows:

The cation exchanger was packed into a column (length 7 cm., diameter 1.4 cm.) by pouring the resin as a suspension in water into a glass tube similar in construction as the columns shown in fig.11. The anion exchanger was poured into a tube similar to A in fig.2, where it settled down to a column 12 cm. long and 1 cm. in diameter. The tubes were supplied with a cotton wool pad at the bottom and a pad of cotton wool was also placed above the surface of the resin. The resins were washed with 2-3 bed

volumes of water before use. The columns were then connected by means of a rubber stopper and the sample (aqueous sol.) to be analysed was introduced to the top of the cation resin from a separating funnel. The solution was allowed to percolate through the resins at a rate of about 0.5 ml. per minute. This was followed by 10 ml. of water and thereafter a further 100 ml. of water in order to ensure that the acids were quantitatively transferred to the anion exchange resin. The effluent from the cation exchanger was neutral (pH-paper) when the columns were separated. The cation exchanger, which had adsorbed the amino acids and the cations originally present in the sample, was washed with NaOH and regenerated with HCl. The chromatographic tube containing the anion exchanger was filled up with water to within 3 cm. from the top and substituted for column A in the gradient elution apparatus shown in fig.2. Column B and the air-inlets were omitted.

(i) Amberlite IRA-400 (Formate). The mixing flask, C, (fig.2) was filled with 200 ml. of water and the reservoir, D, with 200 ml. of 17%(v/v) formic acid. The organic acids were then eluted with formic acid, gradually increasing in concentration. 2 - 3 ml. fractions were collected in test tubes (10 x 1.2 cm.) at a rate of about 1 ml. in 6 minutes.

The contents of each tube were taken to dryness by

evaporation on a water bath as shown in fig.15.

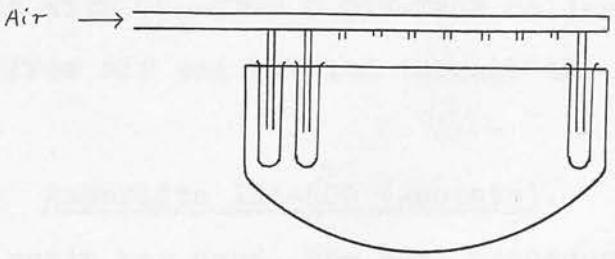


Fig.15

The water bath was supplied with an aluminium cover in which the test tubes were suspended through two rows of circular holes. Corresponding holes were made in a thick-walled rubber tubing (outside diameter 1.3 cm., inside diameter 0.7 cm.) by means of a cork borer, to hold glass tubes (length 8 cm., outside diameter 0.4 cm.). The manifold tubes were suspended in such a way that their openings were approximately 2 cm. from the surface of the liquid in the test tubes. The temperature in the water bath was kept between 50 and 60°C. By blowing air

on to the surface of the solution, evaporation of visible fluid was usually complete in 40-50 minutes and there was then no smell of formic acid. A few drops of water were then added to each tube and the evaporation was continued to dryness. At the end of the drying period, water (1 ml.) was added to each test tube and the solutions were titrated to the phenolphthalein end point with CO_2-free 0.01N-NaOH delivered from a microburette. CO_2-free air was bubbled through the solution during the titration.

(ii) Amberlite IRA-400 (Acetate). When the acetate form of the resin was used, the same procedure as described for the formate resin was followed except for the elution. In this case the elution was started by having water (200 ml.) in the mixing flask C, and 30%(v/v) acetic acid in the reservoir D (fig.2). The fastest moving acids, including succinic acid, were then eluted with acetic acid. Thereafter the contents of the flasks were changed to 10%(v/v) formic acid in flask C and 20%(v/v) formic acid in flask D (fig.2), and the elution continued.

Experiment 9.

A solution (500 ml.) was prepared containing succinic acid (185.0 mg.), malic acid (657.5 mg.) and citric acid (485.0 mg.).

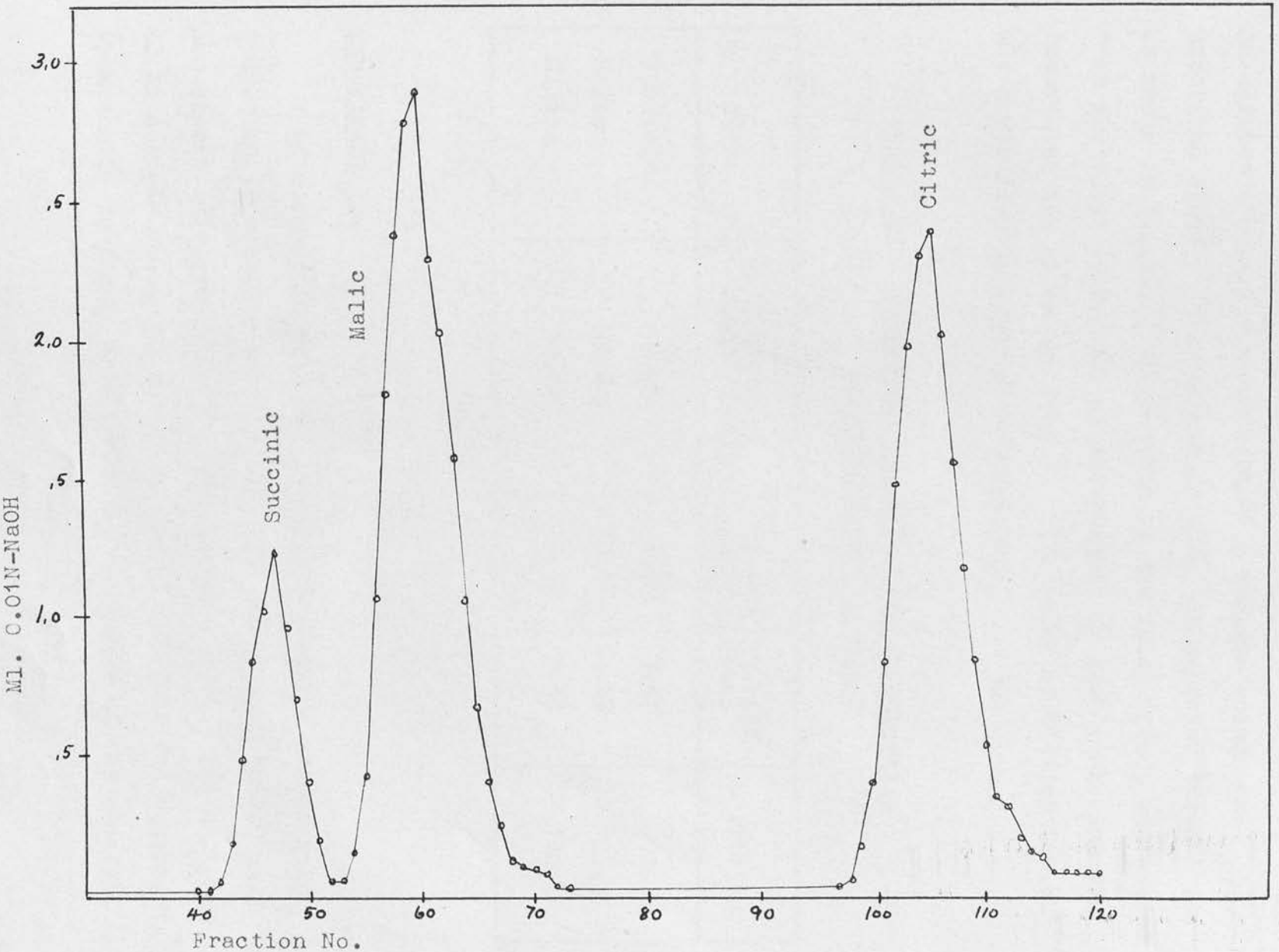


Fig.16. ELUTION OF ACIDS FROM AN AMBERLITE IRA-400 (FORMATE) COLUMN

An aliquot (10 ml.) was analysed on a formate column as described above. The titrations gave the expected three peaks as shown in fig.16. The amounts of the acids found, calculated from the total titres (blank subtracted) of each peak, and the recoveries are given in table 5. The order of elution (60,64) was controlled by paper chromatography.

Table 5. Recoveries of acids from an Amberlite IRA-400 (Formate) column.

Acid	Titre(ml.) 0.00945N-NaOH	mg.acid added	mg.acid found	% recovery
Succinic	6.30	3.70	3.52	95
Malic	19.80	13.15	12.54	95.5
Citric	16.02	9.70	9.69	100

Experiment 10.

A solution (500 ml.) was prepared containing quinic acid (505.0 mg.), succinic acid (558.0 mg.), malic acid (460.0 mg.) and citric acid (520.0 mg.). An aliquot (10 ml.) was analysed on an acetate column as described above, changing the elutriant from acetic to formic acid after the emergence of the succinic

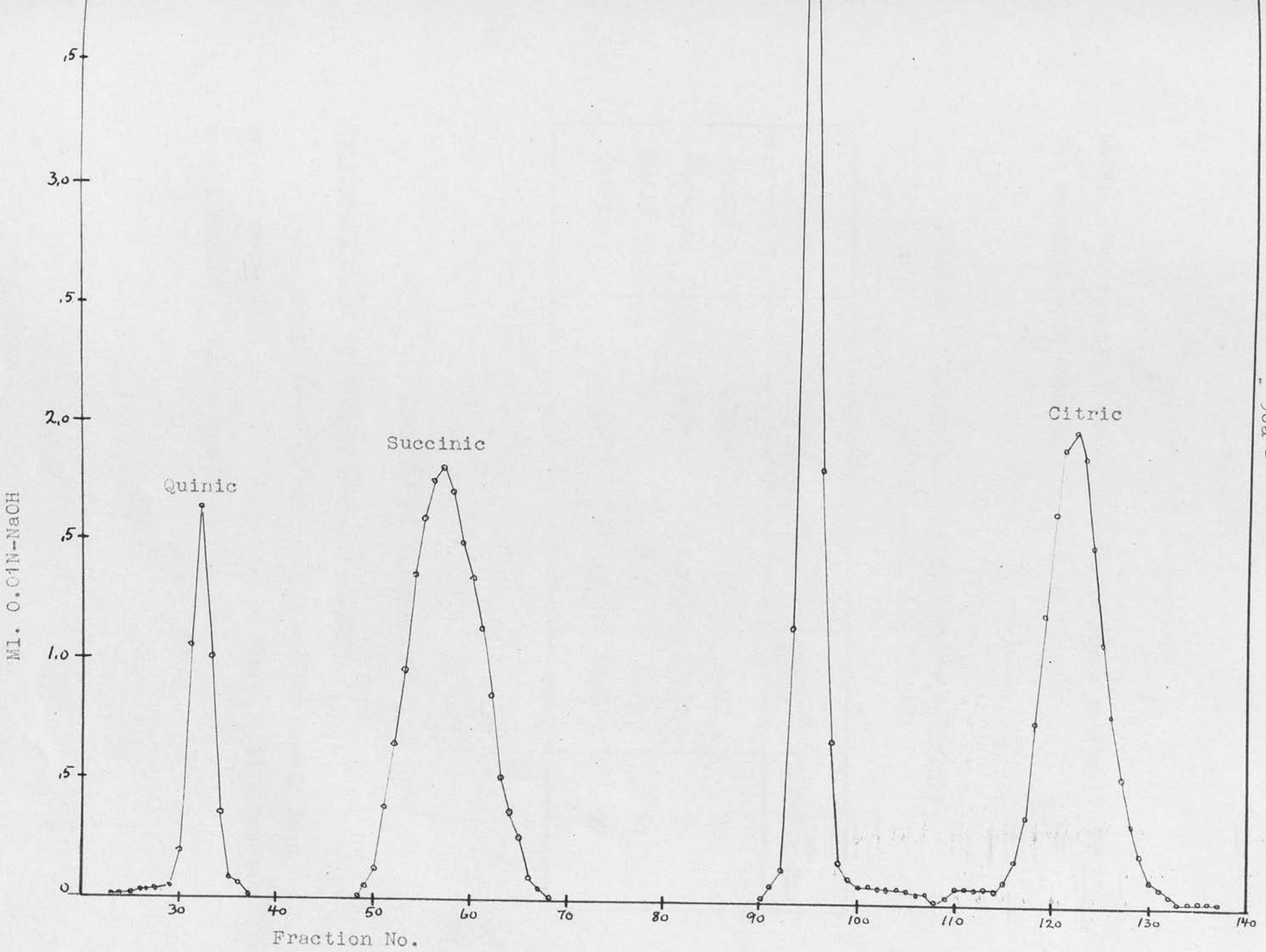


Fig.17 ELUTION OF ACIDS FROM AN AMBERLITE IRA-400 (ACETATE) COLUMN

acid. The result of the titrations is shown in fig.17 and the recoveries in table 6.

Table 6. Recoveries of acids from an Amberlite IRA-400 (Acetate) column.

Acid	Titre(ml.) 0.0110N -NaOH	mg.acid added	mg.acid found	% recovery
Quinic	4.46	10.10	9.42	93
Succinic	16.33	11.16	10.60	95
Malic	12.12	9.20	8.94	97
Citric	14.65	10.40	10.32	99

Analysis of Grass.

Determination of dry matter (D.M.) in grass.

A grass sample (100 g.) was weighed out accurately in a crystallising dish and left in an oven at 95-100°C till constant weight (usually about 8 hours).

Extraction of grass.

The fresh grass sample (100 g.) was suspended in boiling water (500 ml.) in a beaker, boiled gently for 2-3 minutes and allowed to stand with occasional stirring and pressing for 30 minutes. It was then filtered through two layers of cheese-cloth placed in a Buchner funnel. The sample was finally squeezed by hand, transferred to the beaker again and treated twice in the same way with 250 ml. of water. The cloudy extract (a little more than 1 l.) was cooled to room temperature and the pH was determined with a Cambridge pH-meter. This extract was concentrated to about 100 ml., transferred to a 250 ml. graduated flask and made up to the mark. By leaving the flask in the refrigerator, or in running water for 3-4 hours (or overnight), the extract clarified. It was then easily filtered through a dry filter. A drop of chloroform or a few drops of toluene were added to preserve the extract.

Experiment 11.

100 g. of fresh lawn-grass (cut outside the laboratory 10/1/55. Moisture 76.3%) were extracted as described. pH=6.20. A sample of the extract (20 ml.≡1.896 g. D.M.) was passed through the two ion exchange resin columns as previously described. The anion exchanger was in the formate form. The elution was

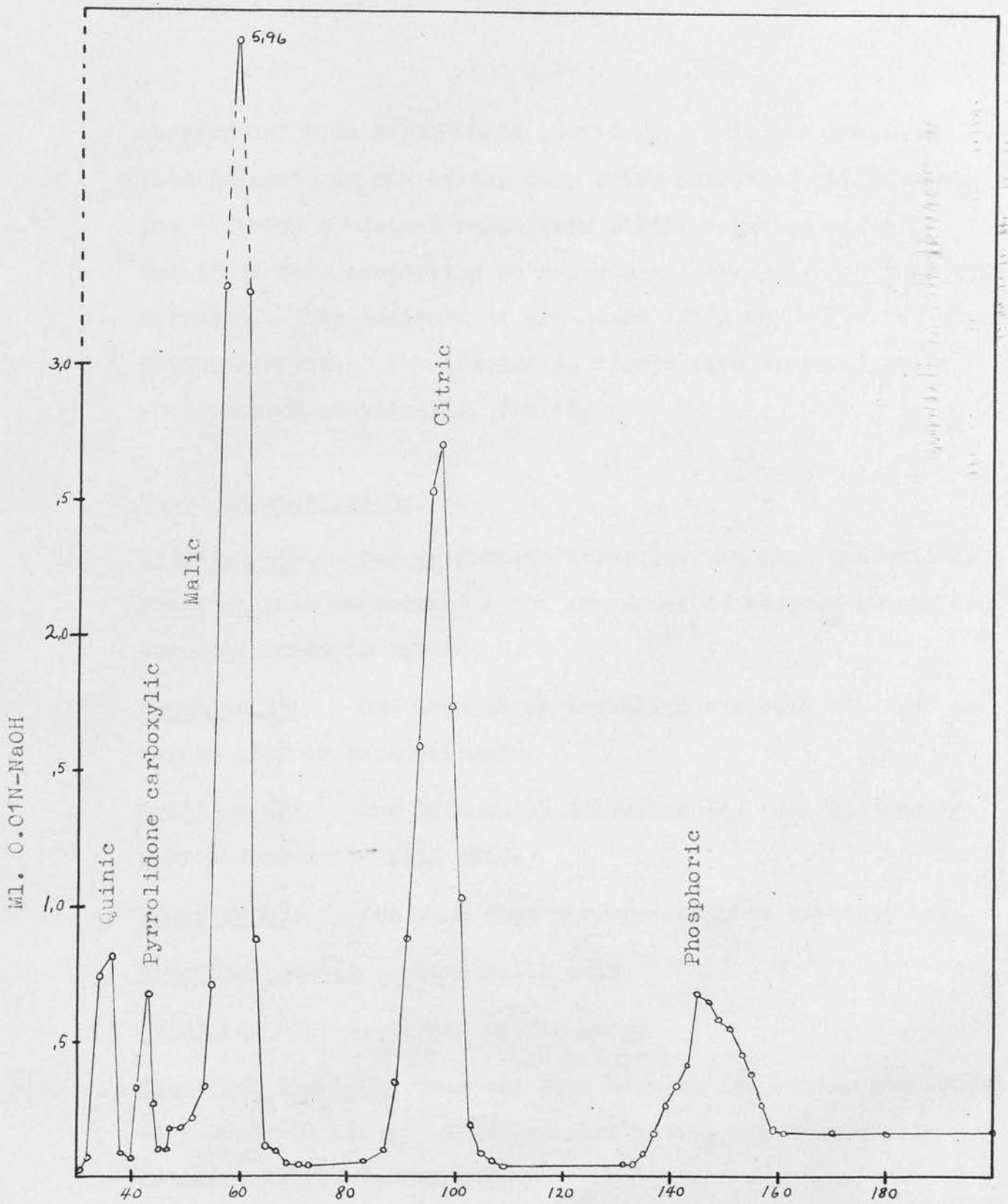


Fig.18 ACIDS IN GRASS ELUTED FROM AN AMBERLITE IRA-400(FORMATE)COLUMN

carried out with formic acid (0-17%v/v). It was observed that in spite of the strong dark brown colour of the extract, the effluent contained relatively little coloured matter. The fractions were evaporated to dryness and every second test tube titrated. The contents of the other tubes were used for paper chromatography. The titrations formed five distinct peaks when plotted graphically, fig.18.

Paper Chromatography.

Fraction 33: One acid which travelled the same distance as succinic acid in solvent A and was detected between quinic and succinic acids in solvent B.

Fraction 35: One acid which travelled the same distance as quinic acid in both solvents.

Fraction 42: One acid which travelled the same distance as pyrrolidone carboxylic acid.

Fraction 47: One weak spot corresponding to succinic acid.

Fractions 56-65: Pure malic acid.

Fractions 97-106: Pure citric acid.

Fractions 138-160: They all gave streaks and spots corresponding to phosphoric acid. A yellow precipitate was formed with ammonium molybdate reagent.

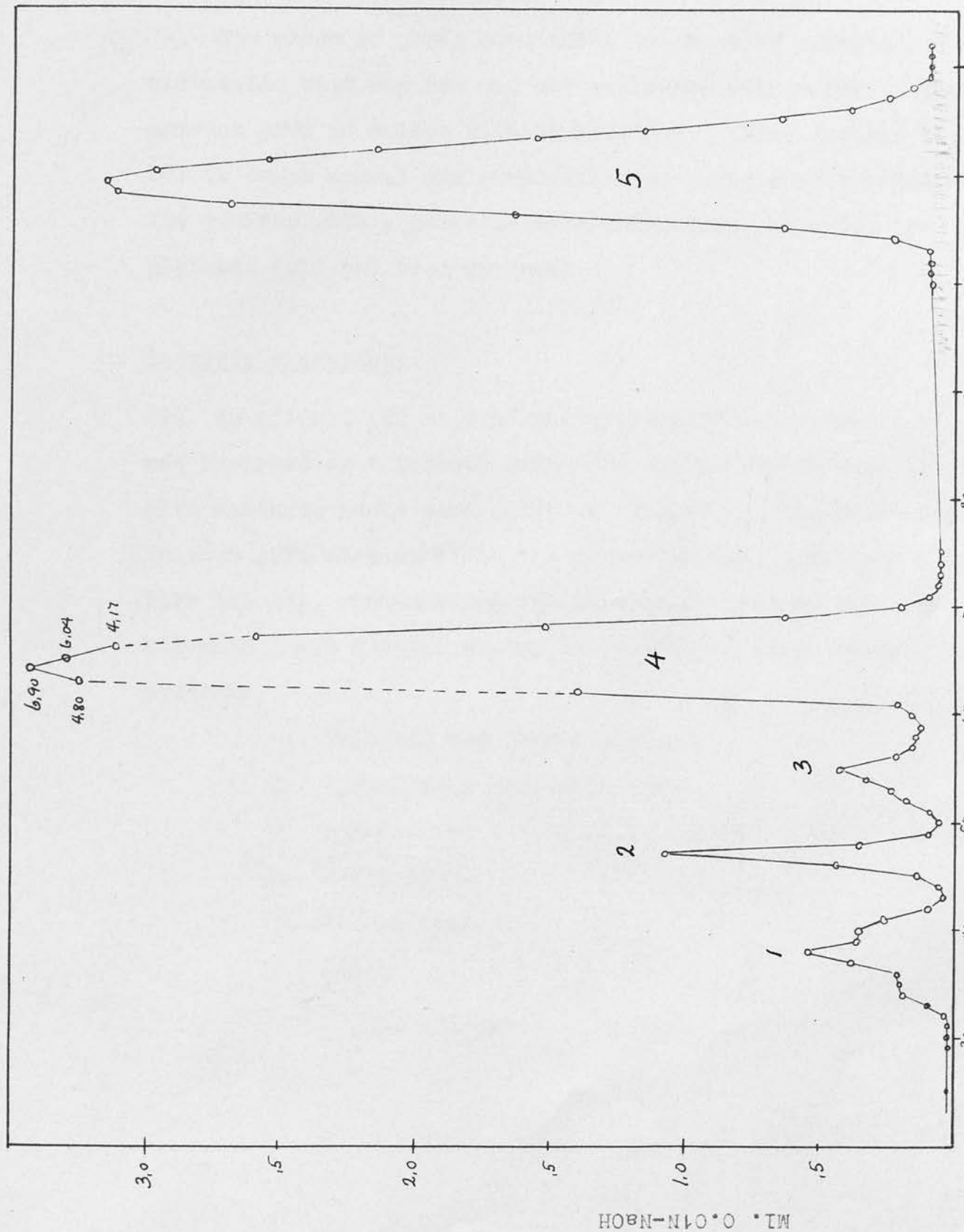


Fig.19 ACIDS IN GRASS ELUTED FROM AN AMBERLITE IRA-400 (FORMATE) COLUMN

The piece of paper containing the spot of pyrrolidone carboxylic acid was cut out and extracted with water. The extract gave no colour with ninhydrine. After boiling with HCl (2 drops conc.) and evaporating to dryness on a water bath, the residue gave a positive ninhydrine test, expected if glutamic acid had been produced.

Recovery experiment.

(i) An aliquot (20 ml.) of the extract from experiment 11 was analysed on a formate resin and all fractions were titrated. Five definite peaks were detected (fig.19). The fractions in each peak were combined and passed through a column of Zeo-Karb 225 (H), concentrated and chromatographed on paper in solvents A and B which showed the following acids to be present:

1. Shikimic and quinic acid.
2. Pyrrolidone carboxylic acid.
3. Succinic acid + trace of another acid (faster).
4. Malic acid.
5. Citric acid.

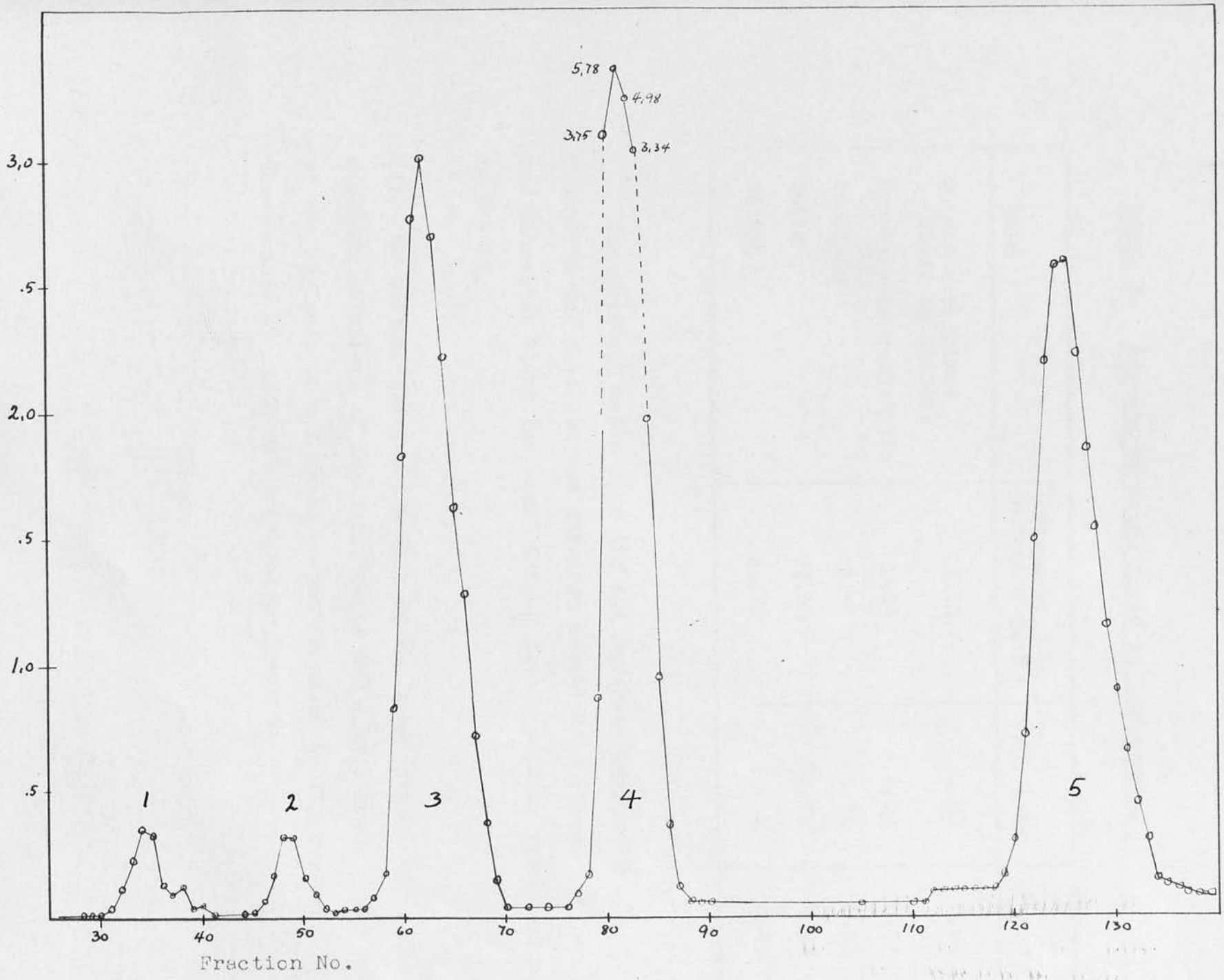


Fig.20 ANALYSIS OF GRASS EXTRACT WITH ADDED SUCCINIC, MALIC AND CITRIC ACIDS

Table 7. Amounts of acids found in lawn-grass.

Acid	Titre(ml.) 0.00945N-NaOH	mg. acid	% D.M.
Quinic + shikimic (calc. as quinic)	2.50	4.54	0.24
Pyrrolidone carboxylic	2.01	2.45	0.13
Succinic	1.50	0.84	0.04
Malic	28.00	17.74	0.93
Citric	21.70	13.12	0.69

Succinic and malic acid did not separate completely. Therefore the division was taken at fraction 59 (Titre 0.13 ml.). The titration blank increased during the experiment from 0.02 ml. to 0.1 ml.

(ii) To another aliquot (10 ml.) of the same extract was added standard solutions of succinic, malic and citric acid. The result of the analysis on a formate column is shown in fig.20, and the amounts of acids found quoted in table 8.

Table 8. Recoveries of acids when added to a grass extract.

Acid	Titre (ml.) 0.00945N-NaOH	mg.acid found	mg.acid present in extract (from table 7)	mg.acid added	% recovery
Quinic	1.32	2.40	2.27	-	
Pyrrolidone	1.02	1.24	1.23	-	
Succinic	17.22	9.60	0.42	9.53	96
Malic	21.91	13.85	8.87	5.26	95
Citric	17.85	10.80	6.56	4.40	97

An aliquot (10 ml.) of the same extract was analysed alone (1) and with addition of standard acids (2). The separation between succinic and malic acid was not good. These two acids were therefore calculated together.

Table 9. Recoveries of acids when added to a grass extract.

Acid	Titre (ml.)		Titre(ml.) of standard acid added	Difference in titre	%recovery
	1	2			
Succinic + malic	14.70	29.06	15.16	14.36	95
Citric	11.0	17.95	6.94	6.95	100

Completeness of extraction.

The material left in the cheesecloth after the final squeezing was subjected to a second extraction with the same amount of boiling water (500 + 250 + 250 ml.). This extract was only slightly coloured. It was concentrated in the usual way. Titration of the fractions showed no acidity except for a slight rise above the blank where the phosphoric acid was eluted.

Lactic acid added to grass extract.

To an aliquot of the grass extract was added lactic acid (8% of D.M.) and the solution analysed on an Amberlite IRA-400 (formate) column. Two drops were withdrawn from each test tube before evaporation and tested for lactic acid (65) by addition of 1 ml. of conc. H_2SO_4 and heating at 85°C. for 2 min. After cooling to 28°C. and addition of a pinch of solid p-hydroxydiphenyl a violet colour appeared when lactic acid was present. After evaporation of the fractions, titration and paper chromatography tests, it was found that the lactic acid had been eluted between quinic and succinic acids. There was a slight overlap between all three acids due to the relatively large amount of lactic acid present. The pyrrolidone carboxylic acid was eluted with the lactic acid and was shown by paper chromatography to be located in the

middle of the lactic acid peak. A similar experiment using elution with acetic acid from a resin generated in the acetate form gave a clean separation between the quinic, lactic and succinic acids. Silage extracts containing an expectedly high proportion of lactic acid were therefore later analysed on a column of Amberlite IRA-400, generated in the acetate form, by elution with acetic followed by formic acid.

Preliminary examination of a silage extract.

A silage extract was obtained from Dr. A.R. Kemble (66). 50 ml. of this (1.7 g. D.M., pH=4.9) were analysed on an Amberlite IRA-400 (formate) column. The lactic acid was located in fractions 34-56. Water was repeatedly added to these fractions during the evaporation process in order to remove as much as possible of the lactic acid. The contents of every second tube were titrated in the first instance and some representative fractions chromatographed on paper in solvents A and B. Thereafter the remaining tubes were titrated. Quinic, pyrrolidone carboxylic and succinic acids were detected. There was no acidity at the point where malic acid was expected, and a slight increase in acidity where the citric acid was expected gave one spot corresponding to malonic acid. The amount of succinic acid was high (0.40% D.M.) compared with the amounts found in fresh grass. The

acid was recovered by means of a cation exchange resin and the melting point (184-6°C.) was the same as that of authentic succinic acid and a mixture of the two.

Identification of the main acids in perennial rye grass.

The chromatographically identical acids from several samples of grass were combined and also those from silage. These were in the form of the salts and were passed through cation exchangers in order to recover the free acids for identification.

Quinic acid fraction (from grass). This was found to contain quinic acid as the major component. However a small spot could be detected on a paper chromatogram corresponding to shikimic acid. On evaporation to dryness, this fraction formed a colourless oil which went hard. It crystallised on addition of dry ether. It was washed with hot acetone, dissolved in aqueous acetone, filtered and evaporated to dryness. M.p.161°C. Authentic quinic acid melted at 163°C. Mixed m.p. 161-62°C.

Succinic acid (from silage). This acid crystallised in a high state of purity when the fractions were evaporated. Its presence could be detected visually by examining the suspected fractions with a magnifying glass. The acid was dissolved in

water, shaken with a little charcoal, filtered and evaporated to dryness. It had m.p. 184°C. alone and mixed with authentic succinic acid.

Malic acid (from grass). A great deal of coloured matter was eluted together with this acid. The acid crystallised when the fractions were evaporated to dryness, but there was always some brownish oil present. The malic acid was dissolved in dry ether and the solution filtered. The ether was removed by evaporation and malic acid crystallised on grinding and drying in desiccator. M.p. 94-96°C. (Authentic malic acid has m.p. 100°C.). When heated on a water bath with 1 ml. of a β-naphthol reagent (2.5 mg. β-naphthol in 100 ml. conc. H_2SO_4) the appearance of a yellowish colour with a blue fluorescence showed the presence of malic acid (65a).

The phenacyl ester was prepared by refluxing the acid with an alcoholic solution of phenacyl bromide for 2 hours (67). The m.p. was 108°C. alone and mixed with an authentic sample.

Citric acid (from grass). This consisted of a brownish syrup which was dissolved in acetone and treated with charcoal. It crystallised when evaporated to dryness in a desiccator. M.p. 145-46°C. (Authentic sample has m.p. 153°C.). Mixed with authentic citric acid the m.p. was 149-50°C. The acid was

converted to ammonium citrazinate (65b) as follows:

A few crystals were treated with 4 drops of thionyl chloride and taken to fumes over a micro burner. Eight drops of conc. aq. ammonia were added and the mixture boiled until about 2 drops of liquid remained. After cooling, 6 drops of conc. H_2SO_4 were added and the mixture was heated until sulphuric acid vapours were given off. It was washed into a test tube and made ammoniacal. The solution was examined in ultra violet light and an intense blue fluorescence was observed.

IV. Determination of oxalic acid in grass.

Perennial rye grass (S 24) was cut at the Bush Estate in February 1955 and examined for oxalic acid by the method described by Baker (68). The grass (60 g. - Moisture 80.1%) was chopped and crushed in a macerator with water (200 ml.) The mixture was made 1N with respect to HCl and boiled for 15 minutes, left to cool and made up to 500 ml. The mixture was shaken, left overnight and filtered through a dry paper. A sample (25 ml.) was left for 5 hours in a stoppered tube with 5 ml. of phosphoric-tungstate reagent (24 g. of sodium tungstate + 40 ml. conc. phosphoric acid made up with water to 1 l.). The mixture

was centrifuged, 20 ml. of the clear solution were transferred to a 50 ml. centrifuge tube, and NH_4OH was added dropwise until the solution was alkaline. 5 ml. of $CaCl_2$ reagent (25 g. anhydrous $CaCl_2$ dissolved in 500 ml. 50% v/v acetic acid + 330 g. sodium acetate trihydrate in 500 ml. water) were added, and the solution left overnight at 5-7°C. A slight precipitate had been formed, which did not seem to be crystalline when examined under a microscope. It was centrifuged and washed with acetic acid (5% v/v saturated with calcium oxalate). The precipitate was dissolved in H_2SO_4 (10% v/v) and the solution heated at 100°C. The amount of oxalic acid was determined by titration with potassium permanganate.

20 ml. deproteinised extract required 0.18 ml. 0.02N potassium permanganate.

This is equivalent to 0.162 mg. oxalic acid.

Oxalic acid in grass = 0.04% of the dry matter.

V. Determination of lactic acid.

Reagents

$CuSO_4$. 4% w/v copper sulphate penta hydrate (Analar).

H_2SO_4. BDH (Analar) sulphuric acid was found to contain no interfering impurities.

p-Hydroxy diphenyl reagent. p-Hydroxy diphenyl (1.5 g.) was dissolved in 0.5% w/v NaOH (100 ml.).

Standard lactic acid S_1. Pure lithium lactate (0.2132 g.) was dissolved in water. 1 ml. conc. H_2SO_4 was added (18b) and the solution diluted to 100 ml. 1 ml. of this solution ≡ 2000 μg. of lactic acid. The standard solution was stored in a refrigerator and was renewed after 3 weeks.

Standard lactic acid S_2. Pure lithium lactate (0.2132 g.) was dissolved in water and diluted to 500 ml. 1 ml. ≡ 400 μg. of lactic acid.

Colorimetric estimation of lactic acid.

The colorimetric estimations were carried out according to Barker and Summerson (69,18b). Standard solutions of lactic acid containing 4, 8, 10 and 20 μg. per ml. were prepared by dilution. 1 ml. aliquots were transferred to pyrex test tubes (20 x 2.3 cm.). The tubes were chilled in an ice bath and 6 ml. of conc. H_2SO_4 were added dropwise to each tube, with vigorous shaking under constant cooling with ice water. The tubes were then placed in an aluminium rack and heated in boiling water for 5 minutes. They were cooled to below 30°C. by leaving at room temperature. (Cooling in ice was found to give erratic results.) One drop of the $CuSO_4$ solution and 2 drops of the p-hydroxy diphenyl reagent were added to each tube, using the same droppers every time. The added reagents were immediately dispersed by shaking

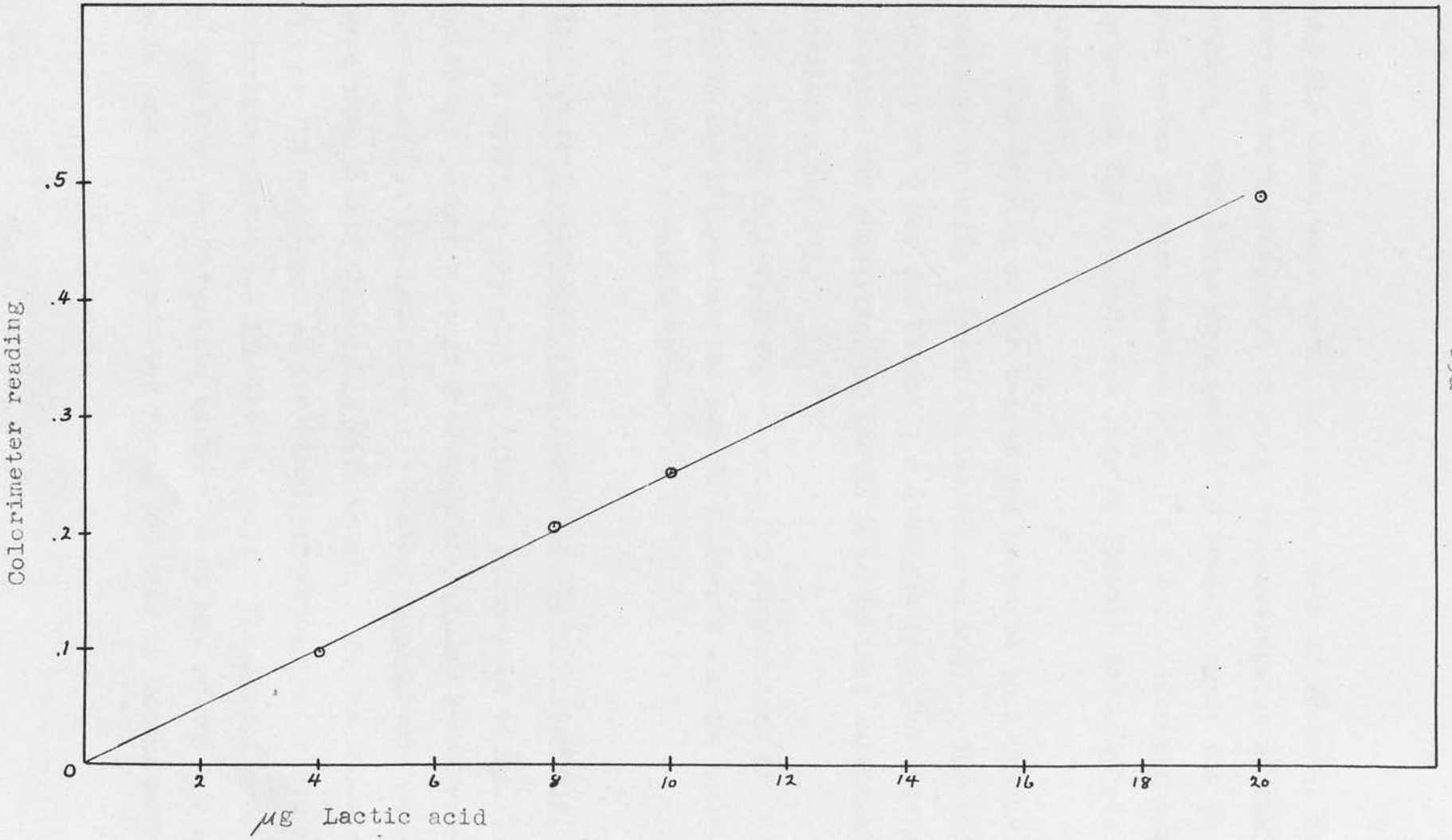


Fig.21 COLORIMETRIC DETERMINATION OF LACTIC ACID

and the tubes were heated in a water bath at 28-30°C. for 30 minutes with occasional shaking, for development of the violet colour. The tubes were heated in boiling water for 90 sec. and cooled to room temperature. A blank containing 1 ml. of water and the reagents was carried through the complete procedure.

The density of the colour was measured on a Unicam colorimeter using a green filter (Ilford 404). The instrument was set at 0 for the blank. A straight line was obtained by plotting the quantities of lactic acid against colorimeter readings. (Fig.21).

In the following experiments the development of the colour was always carried out in duplicate and the average colorimeter readings taken.

Elution of lactic acid from Amberlite IRA-400 (acetate)

A solution (25 ml.) of lithium lactate (≡ 10 mg. lactic acid) was passed through 2 columns containing Zeo-Karb 225(H) and Amberlite IRA-400 (acetate) resins respectively which were then washed thoroughly with water. The Zeo-Karb column was of the same size as described previously while the Amberlite column was shorter (9 cm.). The lactic acid was eluted from the Amberlite resin with 60 ml. of 10% v/v acetic acid into a 1 l. graduated flask and made up to the mark.

1 ml. was withdrawn for colorimetric analysis together with a blank and a standard (10 μg. lactic acid). The colorimeter readings were as follows:

Blank	0
Standard 10μg.	.25
Eluate	.25

Therefore the recovery was 100%.

A separate experiment showed that dilute acetic acid (0.6%) did not give any colour with p-hydroxy diphenyl.

The slope of the straight line (fig.21) varied slightly from one experiment to another. Thus the colorimeter readings for the same standard solution (10 μg. lactic acid) could vary between 0.22 and 0.28. A blank and a standard (10μ g.) were therefore included in each analysis.

Lactic acid in silage.

Experiment 12. A 10 ml. aliquot of the silage extract referred to on p. 93 (≡ 0.1852 g. D.M.) was analysed, in duplicate, as described above.

	Solution	Col. readings	μg. lactic acid	
	Blank	0		
I	Standard S_1	.24	10.0	
	Eluate	.285	12.0	
		.280	11.8	Mean 11.9

To another aliquot (5 ml.) of the same extract was added 10 ml. standard lactic acid, S_2 (≡ 4 mg. lactic acid).

Solution	Col. readings	µg. lactic acid
Blank	0	
Standard S_1	.23	10.0
Eluate	.235	10.2
	.23	10.0

Mean 10.1

Recovery (10.1 - 5.95) = 4.15 = 103.7%

Another experiment was carried out in exactly the same way but on a different silage extract. (See p. 92). Standard S_2 (20 ml.) was added.

II

Solution	Col. readings	µg. lactic acid
Blank	0	
Standard S_1	.279	10.0
Eluate (extract only)	.414	14.9
Eluate (extract + standard S_2)	.432	15.6

Recovery 15.60 - 7.45 = 8.15 = 102%

VI. Determination of volatile acids.

The method of Moyle et al. (37) was adopted for the separation and estimation of butyric, propionic and acetic acids. The acids, dissolved in n-butanol-chloroform (5-95v/v), were added to a column of buffered silica gel and eluted with n-butanol-chloroform, (equilibrated against water). The gel was prepared according to Isherwood (36).

Analysis of a prepared mixture.

Buffer solution. 2M. K_2HPO_4 - 2M. KH_2PO_4 (100-50v/v).

The silica gel (5 g.) and the buffer solution (3 ml.) were mixed and made into a slurry with addition of chloroform (equilibrated against water). This was poured into a glass tube (50 cm. long, 1.3 cm. inside diameter) similar to B, fig.2. The column was swirled by hand and the gel settled down to a uniformly packed column. Equilibrated chloroform (50 ml.) was passed through the column and the side of the tube wiped with a pad of cotton wool on a glass rod to remove loose particles of gel. 0.5 N solutions (50 ml.) of butyric, propionic and acetic acids in chloroform were made up and an aliquot (5 ml.) of each titrated with 0.1000N-NaOH using phenol red. They required 25.35 ml., 26.00 ml. and 28.60 ml. respectively. 1 ml. of CO_2-free water was added and CO_2-free

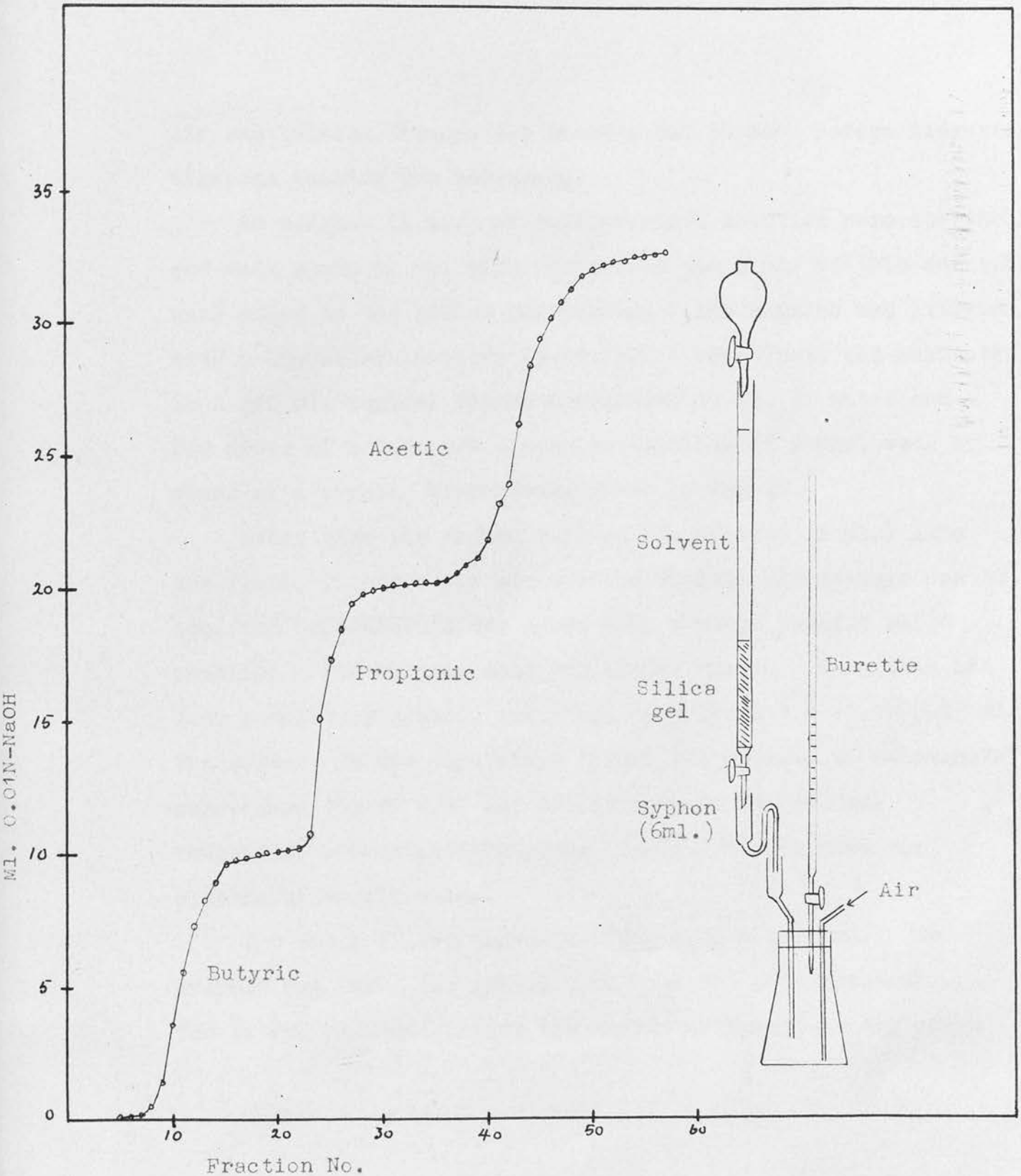


Fig.22 ELUTION OF VOLATILE ACIDS FROM A SILICA GEL COLUMN

air was bubbled through the mixture for 30 sec. before titration. Vigorous shaking was necessary.

An aliquot (5 ml.) of each standard solution were combined and made up to 50 ml. with chloroform and 2 ml. of this solution were added to the silica gel column. The elution was started with n-butanol-chloroform (5-95v/v). The eluate was collected in a 250 ml. conical flask, containing 10 ml. of water and a few drops of a 0.1% w/v alcoholic solution of phenol red, by means of a syphon arrangement shown in fig.22.

Every time the syphon emptied its content (6 ml.) into the flask, CO_2-free air was bubbled through the mixture for 30 sec. and 0.0104N-NaOH was added from a micro burette while shaking. The butyric acid was eluted first. When this had been completely eluted, indicated by a low titre (0.02-0.03 ml.), the solvent in the separating funnel was changed to n-butanol-chloroform (10-90 v/v) and propionic acid was eluted. Thereafter n-butanol-chloroform (30-70 v/v) was used for elution of acetic acid.

The blank titres increased from 0.02 - 0.08 ml. An average was taken for subtraction from the acid fractions. The titres plotted against the number of fractions are shown

in fig.22.

Acid	Total titre(ml.) 0.0104N-NaOH	% Recovery
Butyric	9.57	98
Propionic	9.63	96
Acetic	10.99	100

The three above-mentioned acids were estimated in silage in the following way.

Minced silage (10 g.) was transferred to a steam distillation flask (71). To this was added 20 ml. of water, 10 ml. of a solution of oxalic acid (5%w/v) and 10 ml. of a solution of potassium oxalate (10% w/v). The mixture was then subjected to steam distillation according to Barnett and Duncan (70), 300 ml. of distillate being collected. The distillate was titrated with 0.1N-NaOH and an excess of NaOH (about the same volume as the titre) was added (72). This solution was concentrated under reduced pressure and made up to 25 ml. An aliquot (10 ml.) was evaporated to 0.1-0.2 ml. in a long-necked 100 ml. round-bottomed flask on a boiling water bath, facilitated by a stream of air. After cooling,

a drop of phenol red solution and (A.R.) anhydrous $KHSO_4$ (3-4 g.) was added. The mixture was shaken until a dry pink powder was obtained. The acids were then extracted by shaking with six 4 ml. portions of dry n-butanol-chloroform (5-95v/v) and made up to 25 ml. with this solvent (73). An aliquot (5 ml.) of this solution was then added to the silica gel column and analysed as described.

Changes during conservation.

Definitions and techniques used in the experiments.

Dry matter (D.M.). The dry matter content of the grass was determined as previously described by drying a sample (100 g.) of fresh grass in an oven at 95-100°C.

Total nitrogen (T.N.). This includes all the nitrogen in a particular sample, which can be estimated by the micro-Kjeldahl procedure.

A sample of fresh grass (20-30 g.) was weighed accurately and boiled with 200 ml. conc. H_2SO_4 (N-free) until a homogeneous mixture was obtained. The solution was cooled, made up to 250 ml. and 2 ml. samples were taken for micro-Kjeldahl determination.

Micro-Kjeldahl technique (74). The samples were digested with conc. H_2SO_4 (2 ml.) using 0.2 g. of a catalyst consisting of a mixture of anhydrous K_2SO_4 (80 g.), $CuSO_4$ (20 g.) and sodium selenate (1 g.). The ammonia formed on addition of alkali was distilled into $\frac{N}{70}$ HCl containing Tashiro's indicator (75). Thus 1 ml. of standard acid is equivalent to 0.2 mg. nitrogen. The reagent blank of 0.06 ml. $\frac{N}{70}$ HCl was subtracted from the titrations quoted throughout this work.

Soluble nitrogen (S.N.). This is the fraction of the total nitrogen which can be extracted with boiling water and determined by the micro-Kjeldahl technique. In the following experiments S.N. was determined in an aliquot (1-2 ml.) of the water extract prepared for the estimation of the non-volatile acids.

Volatile nitrogen (V.N.). This is the nitrogen which can be steam distilled from a solution at pH 10.5. It consists mainly of ammonia. V.N. was estimated in the micro-Kjeldahl apparatus by steam distillation of an aliquot (1-2 ml.) of the water extracts after addition of a borax buffer solution (pH 10.5).

Carbohydrate. The sugar analyses quoted in the conservation experiments were carried out by Dr. Wylam, using the extract

used for non-volatile acid determinations, or using a separate water extract of the same sample.

Organic acids. The non-volatile acids were estimated by chromatography on Amberlite IRA-400 anion exchange resin. The elution was always continued for a long time after the phosphoric acid had been eluted in case any slow moving acids might appear. The volatile acids were separated on silica gel. The sum of the titrations (blank subtracted) are given for the individual acids in all cases. The average of duplicate colorimetric estimations are given for the lactic acid determinations. The results are calculated on the basis of the dry weight (% D.M.) of the fresh grass.

Grass used for conservation.

The grass used for conservation experiments was S24 perennial ryegrass unless otherwise stated, and was grown at the Bush Estate. All samples were cut in the morning between 10 and 11.30 a.m., and transferred to the laboratory where they were extracted within 1 hour after cutting. All the samples referred to later had been taken on a sunny day, which had been preceded by two or three sunny days.

Silage No.1.

The changes which take place in silages made in small bottles have previously been found to be essentially the same as in farm silos (76, 77, 78, 79). The grass was minced, unless otherwise stated, mixed well and packed tightly into half pint bottles (about 200 g. in each bottle). The bottles were closed with mercury seals fitted into rubber stoppers, so that gas could escape but no air-borne infection could enter. The fermentation took place under controlled temperature (35°C.). Each bottle was weighed empty, full and before emptying. Before analysis the entire content of the bottle was mixed in a glass dish and portions weighed out for the different determinations.

Experiment 13. Perennial rye grass was cut on the morning of the 25th April 1955. The sample consisted of leaves 3"-4" in height. This experiment was carried out in collaboration with Dr. C.B. Wylam who was investigating carbohydrate changes. Two sets of silages were made. (i) The minced grass was packed into 5 bottles (200 g. in each) and a solution of perennial rye grass fructosan (2.85 g. in 20 ml. of water) was added to each bottle. These were marked FI, FII .....
The amount of fructosan added was subtracted from the dry

weight in the calculations of the results of the analyses.
(ii) 7 bottles were filled with minced grass alone and marked CI, CII .....

The bottles were incubated at 20°C. for the first 3 days. The temperature was then increased to 35°C. and maintained there for the rest of the experiment. 2 bottles were opened for analyses at the same time, 1 control (C) and 1 with fructosan added (F).

Analysis of the fresh grass.

Moisture. 100.0 g. of fresh grass were dried at 95-100°C. to a constant weight of 22.70 g. Therefore the moisture content of the fresh grass was 77.3%.

Total nitrogen. A sample of the fresh grass (25.0 g.) was digested with conc. H_2SO_4. The solution was made up to 250 ml. with H_2SO_4 and two aliquots (2 ml.) were taken for micro-Kjeldahl determinations. These required 5.50 ml. and 5.49 ml. $\frac{N}{70}$ HCl.

Hence T.N. = 550 mg. N/100 g. fresh grass.

Water extraction. A sample of minced grass (100.0 g.) was extracted with boiling water (500 + 250 + 250 ml.) as described

previously. pH = 6.18. This was concentrated and made up to 250 ml.

Soluble nitrogen. Aliquots (2 x 2 ml.) of the water extract were subjected to micro-Kjeldahl determinations. Average titre of $\frac{N}{70}$ HCl = 2.56 ml.

Hence S.N. = 11.5% of T.N.

Non volatile acids. An aliquot (25 ml.) of the water extract was analysed on an Amberlite IRA-400 (formate) column as previously described.

Acid	Fractions	Titre(ml.) 0.0109N-NaOH	mg. acid	% of D.M.
Quinic	20-28	9.55	19.9	0.88
Succinic	34-38	0.60	0.38	0.02
Malic	40-52	36.32	26.5	1.17
Citric	70-95	23.91	16.7	0.73

Repeated analyses of this extract were carried out in order to study the reproducibility of the results. The essential details are given in table 10 (p. 137).

The acids were recovered from a Zeo-Karb 225 (H) column and run on paper in solvents A and B. The quinic acid peak contained traces of shikimic acid. Fractions 30 and 31 were slightly acid (titre = 0.20 ml.). These fractions gave a spot of pyrrolidone carboxylic acid. The malic acid was pure, while the citric acid contained traces of malonic acid.

Silage incubated for 1 day.

Experiment 14. Bottle C I was removed from the incubator.

Weight of grass ensiled	206.0 g.
Weight of silage	206.0 g.

100.0 g. silage were extracted with water as usual and the extract reduced to 250 ml. pH = 6.20.

Non volatile acids. A sample (20 ml.) was analysed on an Amberlite IRA-400 (formate) column.

Acid	Fractions	Titre(ml.) 0.0109N-NaOH	mg. acid	% of D.M.
Quinic	21-29	7.75	16.2	0.89
Succinic	35-39	0.81	0.52	0.03
Malic	40-50	25.87	18.9	1.04
Citric	74-94	18.51	12.9	0.71

Silage incubated for 3 days.

Bottles C II and F I were removed from the incubator, and water extracts (250 ml.) were made of 100 g. of each silage.

Experiment 15. C II. pH = 6.12.

Weight of grass ensiled 208.0 g.

Weight of silage 207.6 g.

Loss 0.4 g.

Soluble nitrogen. A micro-Kjeldahl determination was carried out on 2 ml. of the extract. Titre of $\frac{N}{70}$ HCl = 6.98 ml.

Hence S.N. = 31.6% of T.N.

Non volatile acids. 20 ml. (≡ 1.82 g. D.M.) of the water extract were analysed on an Amberlite IRA-400 (formate) column.

Acid	Fraction	Titre (ml.) 0.0109N-NaOH	mg. acid	% of D.M.
Quinic	18-26	8.39	17.6	0.96
Succinic	32-38	3.38	2.18	0.12
Malic	38-50	20.40	14.9	0.82
Citric	73-90	16.23	11.3	0.62

Experiment 16. F I. pH = 6.34.

Weight of grass + fructosan ensiled 216.5 g.

Weight of silage 216.3 g.

Loss 0.2 g.

Soluble nitrogen. A micro-Kjeldahl determination was carried out on 2 ml. of the extract. Titre of $\frac{N}{70}$ HCl = 6.0 ml.

Hence S.N. = 30.7% of T.N.

Non volatile acids. An aliquot (20 ml.) of the extract (≡ 1.68 g. D.M., corrected for added fructosan) was analysed on an Amberlite IRA-400 (formate) column.

Acid	Fractions	Titre (ml.) 0.0109N-NaOH	mg. acid	% of D.M.
Quinic	21-30	6.78	14.2	0.84
Succinic	39-53	20.95	13.5	0.80
Malic	53-67	4.70	3.42	0.22
Citric	88-105	15.48	10.8	0.64

Both extracts were tested for lactic acid with p-hydroxy diphenyl. There was no lactic acid present.

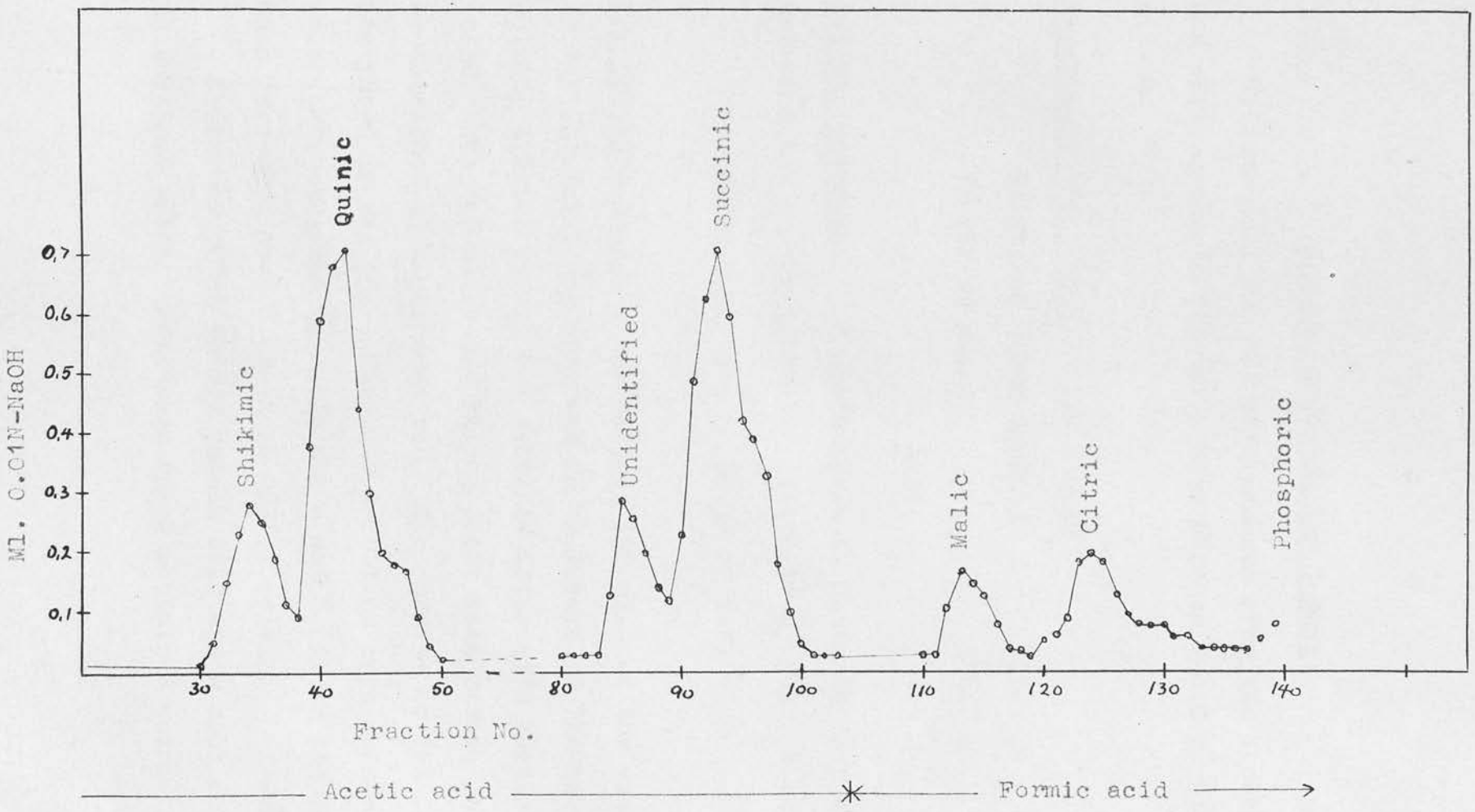


Fig. 23 ACIDS IN SILAGE ELUTED FROM AN AMBERLITE IRA-400 (ACETATE) COLUMN

Silage incubated for 7 days.

Bottles CIII and FII were removed from the incubator, and water extracts (250 ml.) were made of 100.0 g. of each silage.

Experiment 17. CIII. pH = 4.32

Weight of grass ensiled	259.1 g.
Weight of silage	256.7 g.
Loss	2.4 g.

Soluble nitrogen. A micro-Kjeldahl determination was carried out on 2 ml. of the extract. Titre of $\frac{N}{70}$ HCl = 8.66 ml.

Hence S.N. = 39.3% of T.N.

Non volatile acids. An aliquot (10 ml.) of the extract (≡ 0.91 g. D.M.) was analysed on an Amberlite IRA-400 (acetate) column, eluting quinic and succinic acids with acetic acid (0-30% v/v) and malic and citric acids with formic acid (10-20% v/v) as described in experiment 10. The results of the titrations are shown in fig.23. The acid fractions were examined by paper chromatography in solvents A and B, after the acids had been recovered from a Zeo-Karb 225(H) column.

Fractions 34+35 showed quinic acid and a spot corresponding to shikimic acid. Fractions 40-45 contained quinic acid only.

Fractions 85+86 gave a spot of an unidentified acid which travelled at the same rate as tricarballylic acid in solvent B but twice as fast as this in solvent A. Succinic acid was also present in these fractions. Fractions 90-100 contained succinic acid alone. Fractions 111-118 contained pure malic acid and fractions 122-130 pure citric acid. Malonic acid could not be detected. Shikimic and quinic acids were calculated together as quinic acid, and the unidentified acid was included in the succinic acid.

Acid	Fractions	Titre (ml.) 0.0109N-NaOH	mg.acid	% of D.M.
Quinic	30-50	5.02	10.05	1.10
Succinic	83-100	4.76	3.06	0.34
Malic	111-118	0.54	0.39	0.04
Citric	120-133	1.06	0.74	0.08

Lactic acid. A 2 ml. aliquot (≡ 0.182 g. D.M.) was passed through the two resin columns, the Amberlite generated in the acetate form. The lactic acid was eluted in duplicate, with 10% v/v acetic acid (60 ml.) together with quinic and possibly some of the succinic acid. The eluates were made up to 1 l.

and aliquots (1 ml.) of the solutions were analysed as described previously.

Solution	Col. readings	µg. lactic acid
Blank	0	
Standard	.255	10.0
Eluate (a)	.29	11.4
Eluate (b)	.30	11.8

Hence lactic acid = 6.4% of D.M.

Oxalic acid. A sample (60 g.) of the silage (≡ 13.6 g. D.M.) was analysed for oxalic acid as described for fresh grass. No typical calcium oxalate crystals could be seen after precipitation with $CaCl_2$.

20 ml. deproteinised extract required 0.36 ml. 0.02N potassium permanganate. This is equivalent to 0.324 mg. oxalic acid = 0.07% of D.M.

Experiment 18. FII. pH = 4.92

Weight of grass + fructosan ensiled	216.0 g.
Weight of silage	215.5 g.
Loss	0.5 g.

Soluble nitrogen. A micro-Kjeldahl determination was carried out on 2 ml. of the water extract. Titre of $\frac{N}{70}$ HCl = 8.69 ml.

Hence S.N. = 44.5% of T.N.

Non volatile acids. An aliquot (10ml.) of the extract (≡ 0.840 g. D.M., corrected for added fructosan) was analysed on an Amberlite IRA-400 (acetate) column.

Acid	Fractions	Titre(ml.) 0.0109N-NaOH	mg.acid	% of D.M.
Quinic	20-33	4.32	9.05	1.08
Succinic	47-71	13.70	8.8	1.05
Malic	114-126	1.55	1.13	0.13
Citric	126-145	1.53	1.07	0.13

Lactic acid. Duplicate samples (2 ml.) of the water extract (≡ 0.168 g. D.M., corrected for added fructosan) were analysed.

Solution	Col. readings	µg. lactic acid
Blank	0	
Standard	.23	10.0
Eluate (a)	.22	9.6
Eluate (b)	.23	10.0

Hence lactic acid = 5.8% of D.M.

Silage incubated 21 days

Bottles CIV and FIII were removed from the incubator and water extracts (250 ml.) were made from 100.0 g. of each silage.

Experiment 19. CIV. pH = 4.14

Weight of grass ensiled	216.2 g.
Weight of silage	214.8 g.
Loss	1.6 g.

Soluble nitrogen. A micro-Kjeldahl determination was carried out on 2 ml. of the extract. Titre of $\frac{N}{70}$ HCl = 9.97 ml.

Hence S.N. = 45.4% of T.N.

Non volatile acids. An aliquot (10 ml.) of the extract (≡ 0.910 g. D.M.) was analysed on an Amberlite IRA-400 (acetate) column.

Acid	Fractions	Titre(ml.) 0.0109N-NaOH	mg. acid	% of D.M.
Quinic	28-41	5.03	10.5	1.16
Succinic	62-94	4.96	3.2	0.35

Malic and citric acids were not detected.

Lactic acid. Duplicate samples (2 ml.) of the extract (≡ 0.182 g. D.M.) were analysed.

Solution	Col. readings	µg. lactic acid
Blank	0	
Standard	.253	10.0
Eluate (a)	.325	12.9
Eluate (b)	.335	13.3

Hence lactic acid = 7.2% of D.M.

Experiment 20. FIII. pH = 4.58

Weight of grass + fructosan ensiled	216.2 g.
Weight of silage	214.7 g.
Loss	1.5 g.

Soluble nitrogen. A micro-Kjeldahl determination was carried out on 2 ml. of the extract. Titre of $\frac{N}{70}$ HCl = 9.36 ml.

Hence S.N. = 42.6% of T.N.

Non volatile acids. An aliquot (10 ml.) of the extract (≡ 0.840 g. D.M., corrected for added fructosan) was analysed on an Amberlite IRA-400 (acetate) column.

Acid	Fractions	Titre(ml.) 0.0109N-NaOH	mg.acid	% of D.M.
Quinic	43-70	4.03	8.5	1.0
Succinic	100-121	17.78	11.4	1.36
Malic	172-82	2.00	1.46	0.17
Citric	183-192	0.93	0.65	0.08

Lactic acid. Duplicate samples (2 ml.) of the extract (≡ 0.168 g. D.M.) were analysed.

Solution	col. readings	µg. lactic acid
Blank	0	
Standard	.225	10.0
Eluate (a)	.240	10.7
Eluate (b)	.25	11.2

Hence lactic acid = 6.5% of D.M.

Silage incubated for 76 days.

100.0 g. of the contents of bottles CV and FIV were extracted with water. In this experiment and in the following silage experiments an aliquot of the extract was taken for

lactic acid determination before the extract was concentrated.

Experiment 21. CV.

Weight of grass ensiled	228.0 g.
Weight of silage	226.8 g.
Loss	1.2 g.

The water extract was cooled and made up in a measuring cylinder to 1110 ml. pH = 4.10. An aliquot (50 ml.) was set aside for lactic acid determination. (Extract i). The remainder (1060 ml.) was concentrated to 250 ml. (Extract ii).

Non volatile acids. An aliquot (10 ml.) of extract ii (≡ 0.867 g. D.M.) was analysed on an Amberlite IRA-400 (acetate) column.

Acids	Fractions	Titre(ml.) 0.0100N-NaOH	mg. acid	% of D.M.
Quinic	33-60	5.00	9.6	1.11
Succinic	81-95	4.84	2.86	0.33
Malic	126-141	0.97	0.65	0.08
Citric	151-167	0.83	0.53	0.06

Lactic acid. A 10 ml. aliquot of extract i (≡ 0.205 g. D.M.) was analysed. This was the determination quoted under experiment 12. II p. 71 . The eluate was made up to 1 l. as usual and a colorimetric estimation on an aliquot (1 ml.) gave a result of 14.9 μg. lactic acid. Therefore the lactic acid content was 7.3% of D.M.

Experiment 22. FIV.

Weight of grass + fructosan ensiled	216.0 g.
Weight of silage	214.2 g.
Loss	1.8 g.

100.0 g. were extracted and made up to 1160 ml. pH = 4.42. 50 ml. were set aside for lactic acid determination (extract i). The remainder was concentrated to 250 ml. (Extract ii).

Non volatile acids. A 10 ml. aliquot of extract ii (≡ 0.824 g. D.M.) was analysed on an Amberlite IRA-400 (acetate) column.

Acid	Fractions	Titre (ml.) 0.0100N-NaOH	mg.acid	% of D.M.
Quinic	21-33	4.80	9.2	1.12
Succinic	39-62	14.46	8.5	1.03
Malic	110-119	0.93	0.62	0.08
Citric	122-133	0.83	0.53	0.06

Lactic acid. A 10 ml. aliquot (≡ 0.185 g. D.M.) of extract i was analysed. This was the determination quoted under Experiment 12 I p. 70. Colorimetric estimation gave 11.9 µg. lactic acid. Therefore the lactic acid content in the sample was 6.4% of D.M.

Silage incubated for 143 days.

Bottles CVI and FV were removed from the incubator and 100.0 g. of the contents of each bottle were extracted with water. Samples (10 g.) of the silages were weighed out accurately and stored at -20°C. for later volatile acid determination.

Experiment 23. C VI.

Weight of grass ensiled	234.8 g.
Weight of silage	233.2 g.
Loss	1.6 g.

100.0 g. were extracted and made to 1075 ml. pH = 4.34. 50 ml. were set aside for lactic acid determination (Extract i). The remainder was concentrated to 250 ml. (Extract ii).

Soluble nitrogen. A micro-Kjeldahl determination was carried

out on 2 ml. of extract ii. Titre of $\frac{N}{70}$ HCl = 10.60 ml.

Hence S.N. = 51.6% of T.N.

Non volatile acids. An aliquot (10 ml.) of extract ii (≡ 0.8656 g. D.M.) was analysed on an Amberlite IRA-400 (acetate) column.

Acid	Fractions	Titre (ml.) 0.0104N-NaOH	mg. acid	% of D.M.
Quinic	20-30	5.11	10.2	1.18
Succinic	43-49	2.46	1.5	0.17
Malic	102-105	0.80	0.54	0.06
Citric	105-114	0.70	0.46	0.05

Lactic acid. A 10 ml. aliquot of extract i (≡ 0.211 g. D.M.) was analysed.

Solution	Col. readings	µg. lactic acid
Blank	0	
Standard	.220	10.0
Eluate	.255	11.6

Hence lactic acid = 5.5% of D.M.

Volatile acids. A volatile acid determination was carried out on 10 g. of frozen silage as described on p. 74 .

Acid	Titre(ml.) 0.0110N-NaOH	mg. acid	% of D.M.
Butyric	0		
Propionic	0		
Acetic	3.68	2.43	1.3

Experiment 24. F V.

Weight of grass + fructosan ensiled 216.0 g.

Weight of silage 214.1 g.

Loss 1.9 g.

100.0 g. were extracted and made to 1166 ml. pH = 4.40.
50 ml. were set aside for lactic acid determination (Extract i).
The remainder was concentrated to 250 ml. (Extract ii).

Soluble nitrogen. A micro-Kjeldahl determination was carried out on 2 ml. of extract ii. Titre of $\frac{N}{70}$ HCl = 10.21 ml.

Hence S.N. = 52.2% of T.N.

Non volatile acids. A 10 ml. aliquot of extract ii (≡ 0.804 g. D.M.) was analysed on an Amberlite IRA-400 (acetate) column.

Acid	Fractions	Titre (ml.) 0.0104N-NaOH	mg. acid	% of D.M.
Quinic	19-34	4.92	9.8	1.22
Succinic	40-63	16.70	10.3	1.28
Malic	89-99	1.73	1.2	0.15
Citric	102-110	1.43	0.9	0.12

Lactic acid. A 10 ml. aliquot of extract i (≡ 0.180 g. D.M.) was analysed.

Solution	Col. readings	µg. lactic acid
Blank	0	
Standard	.220	10.0
Eluate	.220	10.0

Hence lactic acid = 5.5% of D.M.

Volatile acids. 10 g. of frozen silage were analysed.

Acid	Titre(ml.) 0.0110N-NaOH	mg. acid	% of D.M.
Butyric	0		
Propionic	0.24	0.20	0.12
Acetic	3.40	2.24	1.3

Silage No.2

S24 perennial rye grass was cut at the Bush Estate on August the 29th, 1955. The grass had been sown late and had not reached the flowering stage.

Characteristics of the Grass.

Moisture = 82.2%.

Total nitrogen. A sample of the fresh grass (25.0 g.) was digested with conc. H_2SO_4 and the solution made up to 250 ml. In a micro-Kjeldahl determination an aliquot (2 ml.) of this solution required 6.70 ml. $\frac{N}{70}$ HCl.
Hence T.N. = 670 mg. N/100 g. fresh grass.

Experiment 25. A sample (100.0 g.) of the fresh grass was macerated and a water extract (250 ml.) was prepared in the usual way. pH = 6.22.

Soluble nitrogen. A micro-Kjeldahl determination was carried out on an aliquot (2 ml.) which required 2.12 ml. $\frac{N}{70}$ HCl.
Hence S.N. = 7.9% T.N.

Volatile nitrogen. The steam distillate from a buffered solution (pH = 10.5) of a 10 ml. aliquot of the extract required 0.50 ml. $\frac{N}{70}$ HCl.
Hence V.N. = 0.4% T.N.

Sugars. (determined in the water extract).

Total sugar	9.11% of D.M.
Reducing sugar	2.48% of D.M.
Non-reducing sugar	6.63% of D.M.

Non volatile acids. A 20 ml. aliquot of the extract was analysed on an Amberlite IRA-400 (formate) column).

Acid	Fractions	Titre(ml.) 0.0110N-NaOH	mg. acid	% of D.M.
Quinic	16-23	7.15	15.1	1.06
Succinic	29-32	0.60	0.39	0.03
Malic	33-45	49.79	36.7	2.58
Citric	66-83	12.73	8.98	0.63

A sample of the fresh grass was chopped to ca. 2" lengths with a pair of scissors and packed into half pint bottles, which were closed with mercury seals. The bottles were incubated at 35°C. and their contents analysed after 2 days, 1 week, 12 weeks and 19 weeks. About 10 g. samples were frozen for volatile acid determination. The remainder was macerated and extracted with water in the usual way.

Silage incubated for 2 days.

Weight of grass ensiled	112.1 g.
Weight of silage	111.6 g.
Loss	0.5 g.

Experiment 26. 102.1 g. were extracted and made to 1130 ml. pH = 6.14. 100 ml. were set aside for lactic acid determination (Extract i). The remainder was concentrated to 250 ml. (Extract ii).

Soluble nitrogen. A micro-Kjeldahl determination was carried out on a 2 ml. aliquot of extract ii which required 9.59 ml. $\frac{N}{70}$ HCl.

Hence S.N. = 38.5% of T.N.

Volatile nitrogen. The steam distillate from a buffered solution (pH = 10.5) of a 2 ml. aliquot of extract ii required 1.83 ml. $\frac{N}{70}$ HCl.

Hence V.N. = 7.4% of T.N.

Sugars (determined in extract ii).

Total sugar	2.35% of D.M.
Reducing sugar	0.66% of D.M.
Non-reducing sugar	1.69% of D.M.

Non volatile acids. A 10 ml. aliquot of extract ii (≡ 0.664 g. D.M.) was analysed on an Amberlite IRA-400 (acetate) column.

Acid	Fractions	Titre(ml.) 0.0104N-NaOH	mg. acid	% of D.M.
Quinic	40-60	4.10	8.2	1.23
Succinic	75-113	33.15	20.4	3.07
Malic	124-36	1.75	1.22	0.18
Citric	137-51	0.79	0.53	0.08

Lactic acid. A 10 ml. aliquot of extract i (≡ 0.160 g. D.M.) was analysed.

Solution	Col. readings	µg. lactic acid
Blank	0	
Standard	.220	10.0
Eluate	.055	2.5

Hence lactic acid = 1.6% of D.M.

Volatile acids. The determinations were carried out on 10 g. of frozen silage.

Acid	Titre(ml.) 0.0104N-NaOH	mg. acid	% of D.M.
Butyric	0		
Propionic	-	trace	-
Acetic	2.54	1.59	1.1

Silage incubated for 1 week

Weight of grass ensiled	110.7 g.
Weight of silage	109.7 g.
Loss	1.0 g.

Experiment 27. 97.9 g. were extracted and made to 1083 ml. pH = 5.96.

100 ml. were set aside for lactic acid determination (Extract i). The remainder was concentrated to 250 ml. (Extract ii).

Soluble nitrogen. A micro-Kjeldahl determination on a 2 ml. aliquot of extract ii required 11.84 ml. $\frac{N}{70}$ HCl.

Hence S.N. = 49.7% of T.N.

Volatile nitrogen. The steam distillate from a buffered solution (pH = 10.5) of a 2 ml. aliquot of extract ii required 2.55 ml. $\frac{N}{70}$ HCl.

Hence V.N. = 10.7% of T.N.

Sugars (determined in extract ii).

Total sugar	1.79% of D.M.
Reducing sugar	1.17% of D.M.
Non-reducing sugar	0.62% of D.M.

Non volatile acids. A 10 ml. aliquot of extract ii (≡ 0.633 g. D.M.) was analysed on an Amberlite IRA-400 (acetate) column.

Acid	Fractions	Titre(ml.) 0.0104N-NaOH	mg. acid	% of D.M.
Quinic	15-23	4.44	8.88	1.40
Succinic	28-38	34.53	21.2	3.35

Malic and citric acids were absent.

Lactic acid. A 10 ml. aliquot of extract i (≡ 0.161 g. D.M.) was analysed.

Solution	Col. readings	µg. lactic acid
Blank	0	
Standard	.235	10.0
Eluate	.10	4.2

Hence lactic acid = 2.6% of D.M.

Volatile acids. The determination was carried out on 10 g. of frozen silage.

Acid	Titre(ml.) 0.0104N-NaOH	mg. acid	% of D.M.
Butyric	0.39	0.36	0.25
Propionic	0.20	0.15	0.11
Acetic	3.91	2.44	1.70

Silage incubated for 12 weeks

Weight of grass ensiled	109.2 g.
Weight of silage	108.0 g.
Loss	1.2 g.

Experiment 28. 91.70 g. were extracted and made to 1031 ml.
pH = 5.30.
100 ml. were set aside for lactic acid determination (extract i).
The remainder was concentrated to 250 ml. (extract ii).

Soluble nitrogen. A micro-Kjeldahl determination on a 2 ml. aliquot of extract ii required 11.63 ml. $\frac{N}{70}$ HCl.
Hence S.N. = 52.5% of T.N.

Volatile nitrogen. The steam distillate from a buffered solution (pH = 10.5) of a 2 ml. aliquot of extract ii required 2.52 ml. $\frac{N}{70}$ HCl.
Hence V.N. = 11.3% of T.N.

Sugars (determined in extract ii).

Total sugar	1.37% of D.M.
Reducing sugar	1.22% of D.M.
Non-reducing sugar	0.15% of D.M.

Non volatile acids. A 10 ml. aliquot of extract ii (≡ 0.590 g. D.M.) was analysed on an Amberlite IRA-400 (formate) column.

Acid	Fractions	Titre(ml.) 0.0100N-NaOH	mg. acid	% of D.M.
Quinic	15-23	4.71	9.02	1.53
Succinic	28-35	14.44	8.52	1.44

Malic and citric acids were absent.

Lactic acid. A 10 ml. aliquot of extract i (≡ 0.158 g. D.M.) was analysed.

Solution	Col. readings	µg. lactic acid
Blank	0	
Standard	.25	10.0
Eluate	.045	1.8

Hence lactic acid = 1.1% of D.M.

Volatile acids. The determinations were carried out on 10 g. of frozen silage.

Acid	Titre(ml.) 0.0100N-NaOH	mg. acid	% of D.M.
Butyric	7.69	6.78	4.8
Propionic	2.35	1.74	1.2
Acetic	4.78	2.87	2.02

Silage incubated for 19 weeks

Weight of grass ensiled	110.5 g.
Weight of silage	107.4 g.
Loss	2.1 g.

Experiment 29. 90.0 g. were extracted and made to 1035 ml. pH = 5.66.
50 ml. were set aside for lactic acid determination (extract i). The remainder was concentrated to 250 ml. (extract ii).

Soluble nitrogen. A micro-Kjeldahl determination on a 1 ml. aliquot of extract ii required 5.55 ml. $\frac{N}{70}$ HCl.
Hence S.N. = 48.0% of T.N.

Volatile nitrogen. The steam distillate from a buffered solution (pH = 10.5) of a 2 ml. aliquot of extract ii required 5.60 ml. $\frac{N}{70}$ HCl.
Hence V.N. = 24.4% of T.N.

Sugars (determined in extract ii).
Total sugar 0.30% of D.M.

Non volatile acids. A 10 ml. aliquot of extract ii (≡ 0.6096 g. D.M.) was analysed on an Amberlite IRA-400 (acetate) column.

Acid	Fractions	Titre(ml.) 0.0100N-NaOH	mg.acid	% of D.M.
Quinic	17-25	4.12	7.9	1.30
Succinic	32-35	-	traces	-

Malic and citric acids were absent.

Lactic acid. A 10 ml. aliquot of extract i was analysed. There was no lactic acid present.

Volatile acids (determined on 10 g. of frozen silage).

Acid	Titre(ml.) 0.0100N-NaOH	mg. acid	% of D.M.
Butyric	14.50	12.77	9.0
Propionic	3.87	2.86	2.0
Acetic	4.60	2.76	1.9

Silage No.3

Silage prepared with and without addition of citrate.

Young perennial rye grass, 6"-8" high, was cut on August the 16th, 1955. Moisture 81.5%.

A sample of this grass was left for 24 hours in a covered cardboard box. It was then minced and made into silages in the usual way with about 200 g. grass in each bottle. To one portion was added a solution (5 ml.) of sodium tricitrate (1.40 g. per bottle), while another portion was added 5 ml. water per bottle and made into control silage. The bottles were incubated at 35°C. Analysis of the grass at the stage of ensilage is not available as the extract was lost. The moisture content of the grass prior to ensilage was 82.4%.

Silage incubated for 2 days.

Experiment 30. (Control).

Weight of grass ensiled, including 5 ml. of water	203.2 g.
Weight of silage	202.8 g.
Loss	0.4 g.

100.0 g. were extracted and made to 1100 ml. pH = 4.92.
50 ml. were set aside for lactic acid determination (extract i).
The remainder was concentrated to 250 ml. (extract ii).

Non volatile acids. A 10 ml. aliquot of extract ii (≡ 0.655g. D.M.) was analysed on an Amberlite IRA-400 column.

Acid	Fractions	Titre(ml.) 0.011N-NaOH	mg. acid	% of D.M.
Quinic	18-28	5.45	11.5	1.75
Succinic	36-50	15.03	9.75	1.50
Malic	85-88	0.43	0.3	0.05
Citric	-	0	0	-

Lactic acid. A 10 ml. aliquot of extract i was analysed.

Solution	Col. readings	µg. lactic acid
Blank	0	
Standard	.230	10.0
Eluate	.275	12.0

Hence lactic acid = 7.5% of D.M.

Experiment 31. (citrate added).

Weight of grass + citrate ensiled	203.9 g.
Weight of silage	203.0 g.
Loss	0.9 g.

100.0g. were extracted and made to 1100 ml. pH = 4.90.
50 ml. were set aside for lactic acid determination (extract i).
The remainder was concentrated to 250 ml. (extract ii).

Non volatile acids. A 10 ml. aliquot of extract ii (≡ 0.655g. D.M.) was analysed on an Amberlite IRA-400 (acetate) column. The results were calculated as percentage of the dry weight of the grass ensiled.

Acid	Fractions	Titre(ml.) 0.0110N-NaOH	mg.acid	% of D.M.
Quinic	16-31	5.08	10.7	1.60
Succinic	36-50	17.30	11.24	1.69
Malic	86-89	0.40	0.3	0.05
Citric	-	0	0	-

Lactic acid. A 10 ml. aliquot of extract i (≡ 0.156 g. D.M.) was analysed.

Solution	Col. readings	µg. lactic acid
Blank	0	
Standard	.22	10.0
Eluate	.315	14.3

Hence lactic acid = 9.2% of D.M.

Silage incubated for 22 weeks.

Experiment 32 (Control).

Weight of grass ensiled, including 5 ml. of water	200.0 g.
Weight of silage	197.9 g.
Loss	2.1 g.

100.0 g. were extracted and made to 1000 ml. pH = 4.40.
50 ml. were set aside for lactic acid determination (extract i).
The remainder was concentrated to 250 ml. (extract ii).

Non volatile acids. A 10 ml. aliquot of extract ii (≡ 0.653 g. D.M.) was analysed on an Amberlite IRA-400 (acetate) column.

Acid	Fractions	Titre(ml.) 0.0100N-NaOH	mg. acid	% of D.M.
Quinic	16-22	5.35	10.3	1.58
Succinic	33-42	12.26	7.25	1.11
Malic	-	0	-	-
Citric	-	0	-	-

Lactic acid. A 10 ml. aliquot of extract i (≡ 0.172 g. D.M.) was analysed.

Solution	Col. readings	µg. lactic acid
Blank	0	
Standard	.230	10.0
Eluate	.388	16.9

Hence lactic acid = 9.8% of D.M.

Volatile acids (determined in 10 g. of frozen silage)

Acid	Titre(ml.) 0.0100N-NaOH	mg. acid	% of D.M.
Butyric	-	0	
Propionic	-	trace	
Acetic	6.46	3.88	2.83

Experiment 33 (citrate added)

Weight of grass + citrate ensiled	203.1 g.
Weight of silage	201.0 g.
Loss	2.1 g.

100.0 g. were extracted and made to 1015 ml. pH = 4.48.
50 ml. were set aside for lactic acid determination (extract i).
The remainder was concentrated to 250 ml. (extract ii).

Non volatile acids. A 10 ml. aliquot of extract ii (≡ 0.653 g. D.M.) was analysed on an Amberlite IRA-400 (acetate) column.

Acid	Fractions	Titre(ml.) 0.0100N-NaOH	mg.acid	% of D.M.
Quinic	16-25	5.23	10.1	1.54
Succinic	42-61	12.50	7.38	1.13
Malic	-	0	0	
Citric	-	0	0	

Lactic acid. A 10 ml. aliquot of extract 1 (≡ 0.169 g. D.M.) was analysed.

Solution	Col. readings	µg. lactic acid
Blank	0	
Standard	.230	10.0
Eluate	.365	15.9

Hence lactic acid = 9.4% of D.M.

Volatile acids (determined in 10 g. of frozen silage)

Acid	Titre(ml.) 0.0100N-NaOH	mg. acid	% of D.M.
Butyric	-	0	
Propionic		trace	
Acetic	9.70	5.83	4.16

Silage No.4

Silage prepared with and without addition of malate.

A second growth of S24, perennial rye grass, 3"-5" long was cut on September the 23rd, 1955. The moisture content was 81.8%.

Experiment 34. A sample (50 g.) was extracted with boiling water (pH = 6.10) and the extract made to 250 ml.

Non volatile acids. An aliquot (25 ml.) was analysed on an Amberlite IRA-400 (formate) column.

Acid	Fraction	Titre(ml.) 0.0110N-NaOH	mg.acid	% of D.M.
Quinic	23-38	4.86	10.2	1.13
Succinic	-	0	-	-
Malic	58-78	17.65	13.0	1.43
Citric	86-102	11.27	7.95	0.87

A sample of the minced grass was made into silage in the usual way, each bottle containing 220-240 g. of grass. In this experiment a solution of sodium malate was added to one portion of the grass in such a way that each bottle had an addition equivalent to 0.66 g. of malic acid and 10 ml. of water. 10 ml. of water were added to each bottle of control silage. The bottles were incubated at 35°C.

Silage incubated for 20 days

Experiment 35 (Control).

Weight of grass ensiled, including additive	240.4 g.
Weight of silage	237.5 g.
Loss	2.9 g.

100.0 g. were extracted and made to 1100 ml. pH = 4.24.
100 ml. were set aside for lactic acid determination (extract i).
The remainder was concentrated to 250 ml. (extract (ii).

Non volatile acids. A 10 ml. aliquot of extract ii (≡ 0.635 g. D.M.) was analysed on an Amberlite IRA-400 (acetate) column.

Acid	Fractions	Titre(ml.) 0.0110N-NaOH	mg.acid	% of D.M.
Quinic	20-34	3.89	8.2	1.29
Succinic	55-75	7.63	4.95	0.78
Malic	90-96	0.77	0.57	0.09
Citric	102-113	0.46	0.3	0.05

Lactic acid. A 10 ml. aliquot of extract i (≡ 0.158g. D.M.) was analysed.

Solution	Col. readings	µg. lactic acid
Blank	0	
Standard	.225	10.0
Eluate	.328	14.6

Hence lactic acid = 9.2% of D.M.

Volatile acids (determined in 10 g. of frozen silage)

Acid	Titre(ml.) 0.0110N-NaOH	mg. acid	% of D.M.
Butyric	1.85	1.79	1.3
Propionic	0.18	0.15	0.1
Acetic	6.48	4.3	3.1

Experiment 36 (malate added)

Weight of grass ensiled, including malate solution 227.1 g.

Weight of silage 223.8 g.

Loss 3.3 g.

100.0 g. were extracted and made to 1040 ml. pH = 4.60.

100 ml. were set aside for lactic acid determination (extract i).

The remainder was concentrated to 250 ml. (extract ii).

Non volatile acids. A 10 ml. aliquot of extract ii (≡ 0.662 g. D.M.) was analysed on an Amberlite IRA-400 (acetate) column.

Acid	Fractions	Titre(ml.) 0.0110N-NaOH	mg.acid	% of D.M.
Quinic	18-27	3.86	8.15	1.23
Succinic	59-81	18.65	12.1	1.83
Malic	138-44	0.70	0.5	0.08
Citric	150-60	0.40	0.3	0.05

Lactic acid. A 10 ml. aliquot of extract i (≡ 0.167 g. D.M.) was analysed.

Solution	Col. readings	µg. lactic acid
Blank	0	
Standard	.225	10.0
Eluate	.288	12.8

Hence lactic acid = 7.7% of D.M.

Volatile acids (determined in 10.0 g. of frozen silage)

Acid	Titre(ml.) 0.0110N-NaOH	mg.acid	% of D.M.
Butyric	1.34	1.3	0.94
Propionic	-	0	-
Acetic	6.90	4.55	3.3

Silage No.5

Silage prepared with and without addition of sodium metabisulphite.

Non volatile organic acids were determined in 3 samples of a 6 months' old experimental silage, prepared with and without addition of $Na_2S_2O_5$, supplied by Dr. Stirling. The fresh grass

from which the silage had been made was not analysed. The grass was however cut on May the 1st, 1955, on the same plot as the grass described in Silage No.1.

The moisture content of the fresh grass was 78.6%. 20.0 g. of each silage were extracted with water and the solutions made up to 850 ml. Sugar and nitrogen analyses were carried out on aliquots of this extract. 637.5 ml. of the extract (≡ 15 g. silage) were concentrated to 100 ml. and an aliquot (25 ml.) was used for non volatile acid determinations.

Experiment 37.

1. Silage inoculated with lactobacilli. pH = 3.71.

The acids were separated on an Amberlite IRA-400 (acetate) column.

Acid	Fractions	Titre(ml.) 0.0100N-NaOH	mg.acid	% of D.M.
Quinic	13-29	5.24	10.1	1.25
Succinic	52-58	0.86	0.51	0.06
Malic	83-87	0.86	0.58	0.07
Citric	89-99	1.00	0.64	0.08

2. Silage with addition of sodium metabisulphite (0.2 g. in 50 g. of grass). Not inoculated with lactobacilli. pH = 5.4.

The acids were separated on an Amberlite IRA-400 (formate) column.

Acid	Fractions	Titre(ml.) 0.0100N-NaOH	mg.acid	% of D.M.
Quinic	19-26	4.70	9.0	1.12
Succinic	-	0	0	-
Malic	42-51	7.93	5.32	0.66
Citric	74-86	13.20	8.45	1.05

3. Silage with addition of sodium metabisulphite and inoculated with lactobacilli. pH = 4.16.

The acids were separated on an Amberlite IRA-400 (formate) column.

Acid	Fractions	Titre(ml.) 0.0100N-NaOH	mg.acid	% of D.M.
Quinic	23-35	4.90	9.4	1.17
Succinic	54-56	-	trace	
Malic	82-96	2.06	1.39	0.17
Citric	99-119	13.51	8.65	1.08

Effect of wilting and artificial drying on the organic acids in grass.

Experiment 38. The grass used was the same as that described in Silage No.2. Samples (100.0 g.) were dried under different conditions as follows.

1. Dried in an oven at 95-100°C. (no current of air) for 9 hours.

A boiling water extract (250 ml.) was prepared as usual of the dried sample (17.8 g. D.M.). An aliquot (20 ml.) was analysed on an Amberlite IRA-400 (formate) column.

Acid	Fraction	Titre(ml.) 0.0109N-NaOH	mg.acid	% of D.M.
Quinic	16-24	6.71	14.2	1.0
Succinic	29-33	-	trace	
Malic	34-45	31.60	23.30	1.64
Citric	62-78	8.41	5.87	0.41

Lactic acid. A 2 ml. aliquot of the extract was analysed. There was no lactic acid present.

2. Wilted "moist" for 48 hours. The grass (100.0 g.) was placed in a shallow dish covered with a sheet of filter paper. There was a loss of 16.2 g. of water.

76.9 g. of the "wilted" grass were extracted and made to 1020 ml.

50 ml. were set aside for lactic acid determination (extract i). The remainder was concentrated to 250 ml. (extract ii).

Non volatile acids. A 20 ml. aliquot of extract ii (≡ 1.242 g. D.M.) was analysed on an Amberlite IRA-400 (formate) column.

Acid	Fractions	Titre(ml.) 0.0104N-NaOH	mg.acid	% of D.M.
Quinic	15-22	6.88	13.7	1.1
Succinic	-	0	-	-
Malic	34-43	25.17	17.55	1.41
Citric	60-75	8.78	5.85	0.47

Lactic acid. A 10 ml. aliquot of extract i (≡ 0.161 g. D.M.) was analysed.

Solution	Col. readings	µg. lactic acid
Blank	0	
Standard	.24	10.0
Eluate	.02	0.8

Hence lactic acid = 0.5% of D.M.

3. Drying in a desiccator over P_2O_5.

100.0 g. of fresh grass were dried in vacuo over P_2O_5 for 3 days and weighed at intervals.

Time	Weight
4 hours	84.0 g.
22 "	58.4 g.
26 "	49.9 g.
48 "	37.2 g.
3 days	26.4 g.

The grass still contained 32.6% moisture. A water extract (250 ml.) of the whole sample was prepared.

Non volatile acids. A 20 ml. aliquot was analysed on an Amberlite IRA-400 (formate) column.

Acid	Fraction	Titre(ml.) 0.0104N-NaOH	mg.acid	% of D.M.
Quinic	22-30	8.20	16.4	1.1
Succinic	38-40	-	trace	
Malic	43-53	45.54	31.7	2.20
Citric	76-92	12.56	8.36	0.58

Lactic acid. A 2 ml. aliquot of the extract was analysed. There was no lactic acid present.

Experiment 39. The grass used in this experiment was a portion of the grass used in Silage No.3. (See p. 107).

A water extract (250 ml.) was prepared of 100.0 g. of the fresh grass.

Non volatile acids. A 20 ml. aliquot of the extract was analysed on an Amberlite IRA-400 (formate) column.

Acid	Fractions	Titre(ml.) 0.0110N-NaOH	mg.acid	% of D.M.
Quinic	19-29	9.63	20.3	1.37
Succinic	35-40	0.86	0.56	0.04
Malic	42-59	45.60	33.6	2.27
Citric	80-100	16.21	11.4	0.77

Wilted for 48 hours. A sample (200.0 g.) of the grass was spread out on a sheet of paper and wilted in the laboratory for 48 hours. The weight of the wilted grass was 57.0 g. Drying at 95°-100°C. of 20.0 g. of this showed a moisture content of 36.5%. A water extract (250 ml.) was made of 28.4 g. of the wilted grass.

Non volatile acids. A 20 ml. aliquot was analysed on an Amberlite IRA-400 (formate) column.

Acid	Fraction	Titre(ml.) 0.0110N-NaOH	mg.acid	% of D.M.
Quinic	14-20	7.37	15.5	1.1
Succinic	23-24	-	trace	
Malic	27-37	34.67	25.6	1.78
Citric	60-80	8.17	5.75	0.40

Experiment 40. The grass used in this experiment was a portion of the grass used in Silage No.4, (See p. 112, for analysis of the fresh grass).

Dried in a current of hot air. A sample (71.5 g.) was dried in a current of hot air (about 70°C.) supplied by an electric hairdryer as shown in fig.24.

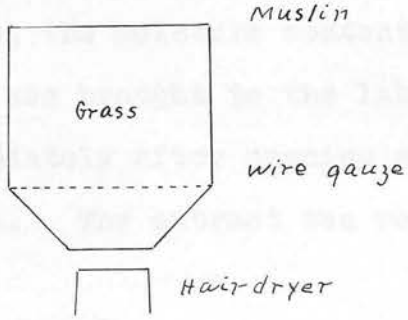


Fig.24

After 1 hour the weight of the sample was 15.0 g. A water extract (250 ml.) was prepared of the entire sample and an aliquot (25 ml.) analysed on an Amberlite IRA-400 (formate) column.

Acid	Fraction	Titre(ml.) 0.0110N-NaOH	mg.acid	% of D.M.
Quinic	16-23	6.38	13.5	1.04
Succinic	35-37	1.40	0.9	0.07
Malic	42-61	23.11	17.0	1.31
Citric	79-107	16.49	11.6	0.89

Experiments with microbe-free grass.

Timothy grass grown in test tubes under sterile conditions was supplied by Dr. Stirling. As only a small sample was available, the moisture content was not determined. The grass (21.1 g.) was brought to the laboratory in sterile containers and immediately after opening was extracted with boiling water (1 l.). The extract was reduced to 100 ml.

Soluble nitrogen. A micro-Kjeldahl determination was carried out on 2 ml. of the extract. Titre of $\frac{N}{70}$ HCl = 2.30 ml.

Hence S.N. = 109mg. N/100 g. fresh grass.

Non volatile acids. A 25 ml. aliquot was analysed on an Amberlite IRA-400 (formate) column.

Acid	Fraction	Titre(ml.) 0.0100N-NaOH	mg.acid	% of fresh weight
Quinic	18-31	0.90	1.73	0.033
Succinic	-	0	-	-
Malic	50-68	7.03	4.72	0.089
Citric	97-112	5.62	3.60	0.068

Wilted sterile grass. The grass was wilted by passing sterile air through the sample for 24 hours. This was carried out by Dr. Stirling under carefully controlled conditions. In this way the sample was reduced in weight from 32.9 g. to 14.1 g. This sample was extracted with boiling water in the same way as the fresh grass.

Soluble nitrogen. A micro-Kjeldahl determination was carried out on 1 ml. of the extract. Titre of $\frac{N}{70}$ HCl = 2.38 ml.
Hence S.N. = 145 mg. N/100 g. fresh grass.

Non volatile acids. A 25 ml. aliquot of the extract (100 ml.) was analysed on an Amberlite IRA-400 (formate) column.

Acid	Fractions	Titre(ml.) 0.0100N-NaOH	mg.acid	% of fresh weight
Quinic	18-27	1.90	3.65	0.044
Succinic	-	0	-	
Malic	45-61	8.93	5.98	0.073
Citric	84-106	12.09	7.75	0.094

Paper chromatography of the acid fractions in solvents A and B showed that the acids mentioned above were present in a pure state.

Organic acids in creeping soft grass (Holcus mollis).

(Preliminary investigation).

A sample of creeping soft grass (Holcus mollis L.) was cut on June the 27th, 1955. The grass had not reached the flowering state. The moisture content was 80.8%. A 250 ml. water extract was prepared of 25.0 g. of fresh grass and a 100 ml. aliquot was analysed on an Amberlite IRA-400 (formate) column in the usual way. The acids eluted in front of the phosphoric acid were found from their order of elution and by

paper chromatography to be quinic, malic and citric acids. Succinic acid was not detected.

Acid	Fractions	Titre(ml.) 0.0109N-NaOH	mg.acid	% of D.M.
Quinic	20-24	3.42	7.22	0.37
Malic	55-63	4.03	2.95	0.15
Citric	90-102	5.00	3.49	0.18

The elution was continued after the phosphoric acid had been eluted, and an acid was eluted well separated from the phosphoric acid in a broad peak. The residue after evaporation was crystalline. The acid gave one spot when run on paper in solvents A, B and D. It travelled the same distance as trans-aconitic acid. The acid melted with decomposition at the same temperature (192°C.) as authentic trans-aconitic acid. An attempt to make the phenacyl ester was not successful. However the acid gave positive test for aconitic acid when converted to ammonium citrazinate (See p. 66).

The amount of trans-aconitic acid found was about 0.7% of D.M.

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D I S C U S S I O N

DISCUSSION

Methods used for isolation of organic acids.

Several chromatographic methods were investigated during attempts to find a satisfactory technique for separating and estimating the organic acids in grass and silage.

Paper chromatography is an ideal method for identification purposes. For quantitative analysis, however, it has the disadvantage that only small amounts of acids can be analysed. A few experiments were carried out in which the acids in a prepared mixture were separated on thick paper chromatograms, eluted with water and then titrated. Formic acid was used as "swamping acid" in the solvent in order to depress ionisation. The paper blank due to incomplete removal of the formic acid was often very high, and varied from strip to strip of the same paper.

In column chromatography a larger amount of acids can be separated and the acids can relatively easily be recovered for identification. Chromatography on silica gel with 0.5N-sulphuric acid as stationary phase and n-butanol-chloroform as mobile phase (36) was tried successfully using prepared mixtures of acids. It was therefore thought that the acids present in grass could be separated by this method.

Finely ground frozen lawn grass was subjected to a mild extraction procedure (36) in which an acidified water extract

was absorbed into silica gel. The acids were eluted in one fraction with n-butanol-chloroform, neutralised with sodium hydroxide and extracted with water. The concentrated water extract of the sodium salts was absorbed into another portion of silica gel and the acids were finally recovered in a solution of tert.-amyl alcohol-chloroform. This solution was chromatographed on a silica gel column and the fractions titrated with sodium hydroxide. When the titres were plotted graphically against the fraction numbers, it only revealed two or three acid peaks (see fig.6, p. 36a). Malic and citric acids have been reported to be the major components of the non volatile acids in grass (11,12). As these two acids did not show up clearly in the titration graph, it was assumed that they had been lost during the extraction. Different extraction and purification methods were therefore investigated. In the free form or as salts of potassium, sodium and calcium the acids are soluble enough to be extracted with water without acidification. If calcium oxalate were present, oxalate would have to be estimated separately.

A water extract was electrodialysed in a three compartment cell (63) in an attempt to separate the acidic fraction from other water soluble components of the grass. There was a vigorous evolution of chlorine in the anode compartment due to chlorides present in the extract, and

much of the coloured material travelled with the acids. The solution was neutralised and concentrated. It was then absorbed in a small portion of silica gel in which the acids were liberated by addition of sulphuric acid. This portion of gel was then transferred to the top of the silica gel column and the acids were eluted with n-butanol-chloroform. 8 or 9 different acids were detected. By paper chromatography in basic and acid solvents, succinic, pyrrolidone carboxylic, malic, citric and probably malonic acids were identified. Malic acid was found to be present in largest amount.

The possibility existed, however, that the chlorine evolved in the anode compartment could have decomposed some of the acids. Citric acid for example in aqueous solution is known to give hexachloroacetone among other products when acted upon by chlorine (80,81). Oxidation may also occur. The method was therefore not considered quite safe.

The same separation of the acidic material from other components in the grass extract can be achieved by means of ion exchange resins. A water extract of fresh lawn grass was freed from cations and amino acids by passing it through a cation exchange resin generated in the hydrogen form (Zeo-Karb 225). Thereafter the anions were adsorbed on a weak anion exchange resin generated in the hydroxyl form (De-Acidite G). The acids were recovered as ammonium salts

by elution with ammonium hydroxide, and then separated on silica gel.

Analyses of aliquots of the same extract, using electrodialysis and ion exchangers for purification of the extract, showed practically the same amount of succinic, malic and citric acids. Comparing the results (see pp.43a,45a), however, two or three more acids were found when electrodialysis had been employed. This was thought to be connected with the chlorine action and oxidation effect at the anode.

Recovery experiments were carried out using the ion exchangers prior to chromatography on silica gel. Aliquots of a water extract of perennial rye grass were analysed alone and with addition of a standard solution containing succinic, malic and citric acids. The recoveries for the individual acids were 83.4, 83.0 and 80.5%. The separation of the acids was good and the peaks obtained by titration were sharp (see pp.48a,49a). When the elution had proceeded for about 4 hours, however, it was noted that a transparent band developed near the top of the analytical column, under the pad of gel in which the acids had been added (See p.43, fig.9). This band would eventually reach a thickness of about 1 cm., and appeared to slow down the flow rate. It might however have a more serious effect. The pad of gel in which the acidic fraction of the grass extract was added to the analytical

column must contain the chloride and phosphate present in the extracts and the sulphuric acid used for liberation of the free acids from their salts. These ions may have retained the water from the solvent so that this was no longer in equilibrium with the stationary phase in the gel. The transparent band might thus have been due to drying out of the gel. (Addition of water gave the gel its normal appearance again.) It was thought possible that this might explain the poor recoveries obtained when grass extracts were analysed, as the top layer of water-rich gel may have held some of the acids.

At this stage a new batch of silica gel was prepared. Chromatography of standard solutions and grass extracts using the new gel did not give sharp, symmetrical titration curves and there was an overlapping of malic and citric acid. The reason for this tailing effect was probably the considerable temperature changes in the room when these experiments were carried out.

The results obtained by chromatography on silica gel and paper confirmed the presence of succinic, malonic, quinic, trans-aconitic, malic and citric acids in grass. Fumaric and pyrrolidone carboxylic acids were also detected in small amounts. The isolation of pyrrolidone carboxylic acid from grass has recently been reported by Ellfolk and Synge (82).

By carrying out the separation of the acids in a constant temperature room, the silica gel method might have led to satisfactory results for the analysis of fresh grass. In silage, however, the large amount of lactic and acetic acid present would have made the estimation of succinic, malic and citric acids difficult because of the low capacity of the partition system. It was therefore thought that the ion exchange resin technique, successfully used for separation of the Krebs cycle acids (60,64), would be more suitable for the present problem.

Although the recommended anion exchange resin Dowex 1 was not available, it was reasonable to believe that similar results could be obtained by using Amberlite IRA-400, as they are both of the strong base type (polystyrene quaternary amine).

An aliquot of the water extract was passed through a cation exchanger (Zeo-Karb 225) generated in the hydrogen form and the anion exchanger generated in the formate or acetate form. The use of a cation exchanger was not necessary for the separation procedure (64), but it was introduced for the removal of coloured material, as most of this was adsorbed by this resin. The acids, adsorbed by the anion exchanger, were eluted with formic acid when the formate form of the resin was employed. 2-3 ml. fractions of the eluate were collected in test tubes and the contents of each tube

evaporated to dryness, avoiding excessive heating. The residues were titrated with sodium hydroxide using phenolphthalein, and the non volatile acids were located in different peaks by plotting the titres against the number of fractions. Recoveries of 95-100% were obtained when a prepared mixture of succinic, malic and citric acids were analysed (see experiments 9 and 10, pp. 54, 56).

Analysis of a sample of fresh lawn grass on an Amberlite IRA-400 (formate) column showed 5 acid peaks on titration of the fractions. The acids were identified by paper chromatography and were found to be shikimic + quinic, pyrrolidone carboxylic, succinic, malic and citric acids in the order of elution. Shikimic and quinic acids were eluted together. However, judging from the size of the spots on the papers, quinic acid seemed to preponderate. It was also evident that malic acid was present in by far the largest amount of any of the acids, followed by citric acid (see table 7).* Phosphoric acid was eluted after citric acid.

Small amounts of fumaric acid were detected by paper chromatography when perennial rye grass was analysed on silica gel. This acid was not detected later when rye grass and silage were analysed by the ion exchange resin technique. Glycollic, glyoxylic, glyceric, pyruvic, oxalic and α-keto-glutaric acids were reported by Palmer (64) to be partially

* p. 60

or wholly lost during the evaporation stage. As these acids were not detected by chromatography on silica gel and paper, they were considered not to be present in the grass in significant quantities.

Analysis of the grass extract alone and with addition of a standard solution containing succinic, malic and citric acids, showed a recovery of 96, 95 and 97% of these acids. It will be noted from figs. 19 and 20 (pp.59a,60a) that the separation of succinic and malic acid was not complete when the amount of succinic acid was of the order of 1 mg., but when 10 mg. succinic acid were eluted, there was no overlapping.

The extraction method used throughout this work involved gentle boiling of the grass for 2-3 minutes three times and squeezing in a cheesecloth. In this way all the organic acids were extracted. A further extraction of the grass sample showed a trace of phosphoric acid only.

Usually the determinations were carried out immediately after extraction, but repeated analysis showed that the acids did not alter in amounts when the extracts were stored 2-3 months in the refrigerator in the presence of toluene or chloroform.

It was found that lactic acid was eluted together with pyrrolidone carboxylic acid and when present in the large

amounts found in silage, it overlapped succinic acid. Nevertheless, it was found that the lactic acid could be removed completely from the fractions by prolonged evaporation and frequent addition of water, as it slowly volatilizes in steam.

The use of the anion exchanger generated in the acetate form gave better separation of the acids eluted before the malic acid, when the elution was carried out with acetic acid. When the succinic acid had been eluted, the elutriant was changed to formic acid. The acetic acid in the resin was then displaced before malic, citric and phosphoric acids were eluted. The separation of malic and citric acids was not so sharp, but usually a blank titre was obtained between the two acids. (See fig.17, p. 56a). Analysis of a prepared mixture of quinic, succinic, malic and citric acids gave recoveries of 93, 95, 97 and 99%.

Busch et al.(60) found that citric acid contained only two titratable acid groups after emergence from a column of Dowex 1 anion exchange resin. On the other hand complete recoveries were obtained when the fractions were analysed colorimetrically. Palmer (64) has reported 98-100% recovery by titration of citric acid when eluted from short columns of Dowex 1. When longer columns were used, however, recoveries of citric acid varied between the limits of 60 to 90%.

In the present work essentially complete recoveries of citric acid based on titration after elution were obtained from a column of Amberlite IRA-400, 0.78 sq. cm. and 12 cm. in length.

Some of the analyses of grass and silage extracts were repeated in order to check the accuracy of the method. Thus when 25 ml. aliquots of the fresh grass extract mentioned in experiment 13 (p. 80) were analysed three times, the results were as shown in table 10.

Table 10. Reproducibility of results.

Acid	Titre(ml.) 0.0109N-NaOH			% of Dry Matter			Deviation in % from mean value
Quinic	8.68	9.55	9.77	0.80	0.88	0.90	5.9
Succinic	0.54	0.60	0.70	0.015	0.017	0.020	14.3
Malic	34.20	36.32	37.62	1.10	1.17	1.21	4.8
Citric	23.90	23.91	25.00	0.73	0.73	0.77	2.7

The large percentage differences in the succinic acid figures are probably due to experimental error because of the small volume of alkali required in relation to the blank. When analysing silage extracts where the amount of succinic

acid was larger, the reproducibility of the results was satisfactory. Thus duplicate analyses of the silage extract quoted in experiment 27 (p.101) gave 3.35 and 3.26% for succinic acid and 1.40 and 1.35% for quinic acid. Sampling errors are not included in the figures above. These may be considerable in farm silages, but will be very small in laboratory experiments, where at least half of the material is extracted.

During analyses of fresh perennial rye grass and silages made from this grass the non volatile acids eluted were checked by paper chromatography. The order of elution from the Amberlite IRA-400 column was quinic (+ shikimic), pyrrolidone carboxylic (+ lactic), succinic, malic, citric and phosphoric acids. Quinic, succinic, malic and citric acids were also identified by mixed melting points of the acids or derivatives and authentic samples. The elution was frequently continued for 100-200 fractions beyond phosphoric acid without detecting other acids. Shikimic acid was always found by paper chromatography to be present in small amount in the quinic acid peak. Further identification of this acid was not attempted. Its isolation from grass has been reported by Hulme and Richardson (16). However, the first peak was calculated as quinic acid only. Malonic acid could often be found in small amounts when the citric acid

fraction was chromatographed on paper. As it was not always found, or found in very small amounts compared with the amount of citric acid, as judged by the size of the spots, this fraction was calculated as citric acid only.

In creeping soft grass (holcus mollis), quinic, malic and citric acids were present in smaller amounts than in rye grass. A relatively large amount of trans-aconitic acid was found however. The cis-aconitic acid had probably been present in the grass originally and had been transformed into the trans-form by heating of the extract (83). In analyses of silages a small amount of an unidentified acid was eluted before succinic acid.

The method was considered accurate enough for the detection of significant changes in the amounts of the non volatile acids mentioned above during conservation of the grass. It was evident, however, that lactic acid and the fatty acids, which are produced in silage fermentation, would have to be estimated by other methods.

The determination of lactic acid in silage has always been a matter of some difficulty. By the older methods (18c,84) the extract was freed from protein and carbohydrate and the lactic acid present oxidised to acetaldehyde which was distilled into standard sodium bisulphite. This was treated with excess iodine which was then titrated with sodium

thiosulphate. Recoveries of lactic acid from silage by these methods were of the order of 85%. The technique of Barker and Summerson (69) as modified by Barnett (18b) has probably been the method most used for lactic acid estimation in silage in recent years. According to this method, protein and other interfering substances are precipitated by addition of calcium hydroxide and copper sulphate. The lactic acid is then estimated colorimetrically utilising the colour reaction between p-hydroxy diphenyl and acetaldehyde formed by oxidation of the lactic acid.

Busch et al.(60) stated that lactic acid emerged quantitatively from a Dowex 1 column. Therefore an attempt was made to purify the lactic acid sample by the same technique as that used for the non volatile acids. There was no loss, as determined by titration, when a solution of lactic acid was boiled for 10 minutes. The water extract prepared by gentle boiling of the silage could therefore be considered to contain all the lactic acid present in the silage. Aliquots of the same extract as used for non volatile acid estimation were therefore subjected to the ion exchange resin treatment, the Amberlite IRA-400 being generated in the acetate form. The lactic acid was then eluted with 10% v/v acetic acid and the eluate diluted with water. This solution was used for the colorimetric determination with sulphuric acid and p-hydroxy

diphenyl. 100% was recovered when a standard solution of lithium lactate was analysed by this procedure. When silage extracts with and without addition of a standard lithium lactate solution were analysed, the calculated recoveries were slightly above 100% (102-103%). Quinic, shikimic, pyrrolidone carboxylic and some of the succinic acid present in the sample were eluted together with the lactic acid. It was found, however, that these acids did not give any colour with p-hydroxy diphenyl when treated according to the procedure for lactic acid.

Acetic acid in the appropriate dilution did not give a colour with the reagents.

The methods most widely applied to the estimation of fatty acids in silage have been based upon steam distillation (85,86,87). This permits the calculation of the total volatile acids present, and the amounts of acetic and butyric acids by application of distillation formulae (88,89). In recent years, however, chromatographic methods have been applied to the examination of the volatile acids in silage (18d,90). In the present work acetic, propionic and butyric acids were separated on a silica gel column (37) after isolation from the silage by steam distillation.

Oxalic acid was determined by precipitation of the calcium salt from an acidified water extract and titration with

potassium permanganate in hot sulphuric acid solution (68).

Non volatile acids in grass and conservation products.

The methods outlined above were applied to perennial rye grass S24 strain at different stages of growth from April to September 1955, and to the products obtained by different conservation methods.

Fresh grass. The grass was extracted within 1-2 hours after cutting. Volatile acids and lactic acid were not detected in the fresh grass. The amount of non volatile acids as percentage of the dry matter varied throughout the summer as shown in table 11.

Table 11. Non volatile acids in perennial rye grass S24 strain as percentage of dry matter.

	Grass sown 8.8.53	Grass sown 19.5.55		
Date of cutting	25.4.55	16.8.55	29.8.55	23.9.55
Quinic acid	0.88	1.37	1.06	1.13
Succinic acid	0.02	0.04	0.03	0
Malic acid	1.17	2.27	2.58	1.43
Citric acid	0.73	0.77	0.63	0.87

The grass cut in April consisted of the early spring growth from the established roots, the previous crop having been cut in November 1954. The two August samples were mature grass which had not yet reached the flowering stage. The September sample was a 38 days old aftermath.

Malic and quinic acids were always present in largest amount and the total organic acid was 3-4% of the dry matter. The amount of acid estimated as quinic acid (probably always contaminated with shikimic acid) was relatively constant throughout the summer. Succinic acid, being present in small quantities, could not be determined accurately enough to detect small variations. It is evident, however, that malic acid increased in amount during the growing period, which is in agreement with the results obtained by Davies and Hughes(11). The changes in citric acid content were not significant, although it seemed to decrease towards the end of the season considering the two August samples. However, too few samples were analysed to get an accurate picture of the seasonal variations.

Silage

In order to study the changes taking place in the organic acid contents during fermentation in silage, the grass was packed into small bottles which were incubated at 35°C. and

removed at intervals for analysis. Thus it is assumed that the fermentation takes the same course in all the bottles so that the contents of one bottle represents the stage of the silage at a certain time.

No addition.

Grass ensiled on August the 29th (Silage No.2) resulted in a product of poor quality as indicated by high pH, high butyric acid and high ammonia content. One bottle of this silage was taken for analysis after two days, one week, twelve weeks, and nineteen weeks. The pH dropped slowly from 6.22 to a minimum of 5.30 after 12 weeks, and rose again to 5.66. The protein breakdown was followed by determination of soluble nitrogen and volatile nitrogen. The soluble nitrogen increased from 7.9% of the total nitrogen to 52.5%, and the volatile nitrogen was 24.4% of total nitrogen after 19 weeks, indicating that protein breakdown had continued beyond the amino acid stage. Total water soluble carbohydrate was 9.11% of the dry matter in the fresh grass and 0.3% in the 19 week silage.

Due possibly to insufficient carbohydrate, the pH did not drop far enough to prevent the action of the butyric acid-forming microorganisms. Thus the final result was a formation of butyric acid instead of the lactic acid essential for a good silage.

It is seen from figs. 25 and 26 that the small amount of lactic acid formed in the beginning of the fermentation had disappeared after 19 weeks and large quantities of butyric acid had been produced. This is generally found to occur in high pH silages due to the presence of the lactate attacking organisms Clostridium butyricum and Cl. tyrobutyricum (20a,91).

Quinic acid increased slightly in amount and seemed to remain constant. Succinic acid, however, which was present in very small amount in the fresh grass, increased rapidly to over 3% of the dry matter and had almost completely disappeared again after 19 weeks. Malic and citric acids both disappeared during the first week of the fermentation.

Fructosan addition.

A silage experiment was carried out in which rye grass fructosan was added to one portion of the grass and a control silage was made with another portion to which no addition was made. (See Silage No.1). The grass was minced and ensiled in bottles maintained at 20°C. for 3 days and thereafter at 35°C. One bottle of each series was analysed at intervals. This grass, cut on April 25th, contained 20% water soluble carbohydrate calculated on a dry matter basis (92) and a "good" silage with no butyric acid was obtained.

During the first 3 days at 20°C. the pH did not drop in

Fig.27 NON-VOLATILE ACIDS IN CONTROL SILAGE (SILAGE NO. 1)

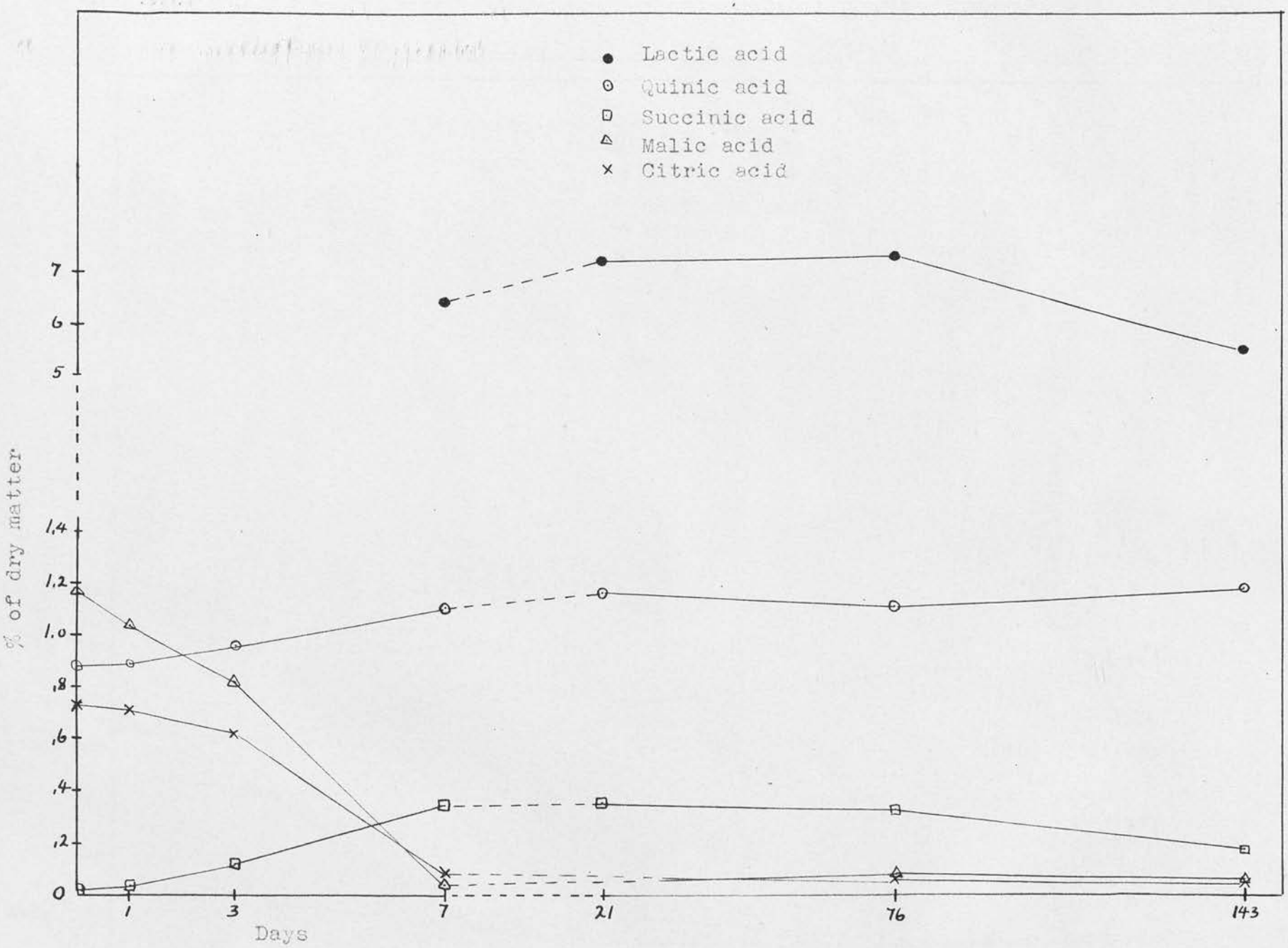
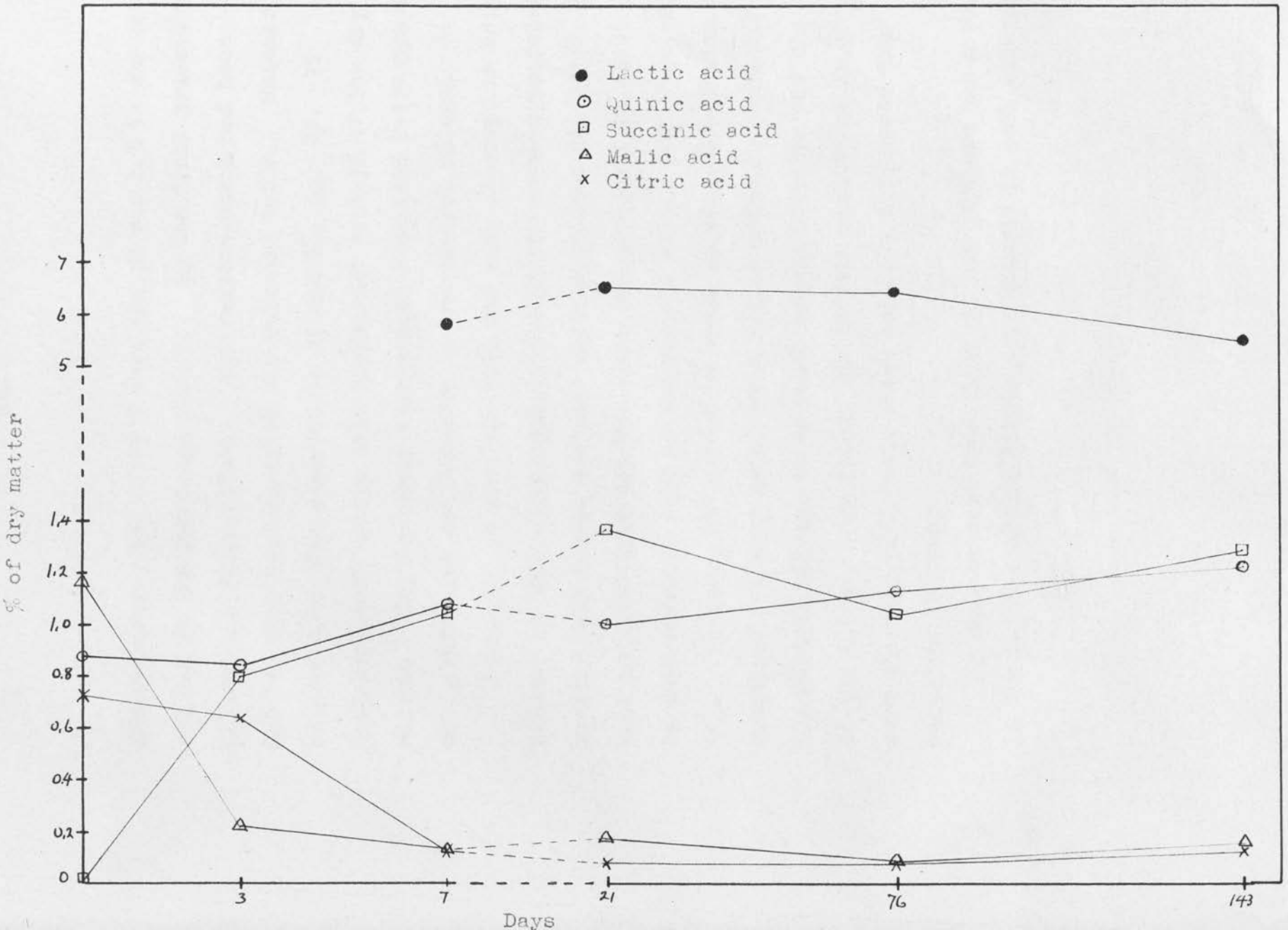


Fig. 28 NON-VOLATILE ACIDS IN FRUCTOSAN SILAGE (SILAGE No. 1)



either series, but after 7 days the pH was 4.3 in the control and 4.9 in the fructosan silage. In the final product pH was 4.3 and 4.4 respectively. The samples which had been incubated for 143 days were analysed for volatile acids. Neither contained butyric acid (See experiments 23 and 24, pp. 93, 95). Both contained acetic acid equivalent to 1.3% of the dry matter. The fructosan silage also contained 0.12% propionic acid, which was not detected in the control silage.

Formation of succinic acid was also observed in both silages in this experiment, although the amounts produced were smaller than in the previous experiment (see fig.26). It will be noted that the fructosan silage contained about 3 times as much succinic acid as the control silage (see figs. 27 and 28). However, the silage to which an aqueous solution of fructosan had been added had a higher moisture content than the control silage, as an equal amount of water was not added to the latter. Therefore the initial conditions in the silages were slightly different, which may have influenced the bacterial activity.

As will be seen from figs. 27 and 28 there was a rapid drop in the malic acid and citric acid content in both silages.

Silages were prepared with addition of malate or citrate in order to study the breakdown of these two acids. In both cases control silages were prepared from the same grass sample as the silage to which addition was made.

Malate addition. Sodium malate equivalent to 1.4% malic acid was added to grass cut on September 23rd (see table 11) so that the malic acid content in the starting material was 2.8% of the dry matter.

After 20 days incubation the control silage contained 0.78% succinic acid and 9.2% lactic acid (see Silage No.4). There was a little butyric acid present. The pH had dropped to 4.24.

In the malate silage the pH dropped to 4.60 and there was 1.8% succinic, and 7.7% lactic acid present. Malic and citric acids had disappeared almost completely in both silages and there was no significant difference in the volatile acid figures.

Although detailed analyses are not available, it became clear that the malate silage deteriorated after further incubation, since a bottle opened after 2 months had pH = 5.3 and gave a strong smell of butyric acid.

It is known that certain bacteria in silage, e.g. some lactobacilli, decarboxylate malate which suggests a possible

explanation for the deterioration of the malate silage. The liberation of cations will lead to a rise in pH. This will favour the activity of the butyric acid-forming microorganisms, and carbohydrate and the lactic acid formed will be fermented to butyric acid.

The control silage had remained at pH 4.2 after 2 months, indicating that the product was of good quality.

Citrate addition. Sodium tricitrate was added to grass which was then ensiled in the usual way. The addition was equivalent to a citric acid content of 2% of the dry matter of the grass. After 2 days incubation at 35°C. the pH had dropped to 4.9 both in the control and in the citrate silage. There was a considerable increase in succinic acid in both, and malic and citric acids had disappeared.

There was more lactic acid in the citrate silage (9.2% D.M.) than in the control (7.5%). After 22 weeks the pH was 4.40 in the control and 4.48 in the citrate silage. The succinic acid content was the same in both (1.1%) and there was no significant difference in the lactic acid figures. There was no butyric and only a trace of propionic acid in the silages. The citrate silage, however, contained much more acetic acid than the control.

From these experiments it is evident that there is a rapid disappearance of malic and citric acids during the silage fermentation. Similar decreases in malic and citric acids have been reported to occur in cider fermentation (93) as brought about by lactobacilli. These organisms also converted quinic acid to dihydroshikimic acid. In the present work, further study into the behaviour of quinic acid was not attempted. The conversion to dihydroshikimic acid by certain lactobacilli, commonly found in cider, has been reported to take place under conditions of reduced oxygen tension (94), which are similar to conditions in silage. It is therefore possible that changes of this kind have been overlooked with the methods used. Succinic acid seems to appear in varied amounts in silage. The presence of this acid has been reported previously in silage (95) and under similar conditions of fermentation in cider (93).

Drying and wilting of grass.

In order to study the changes which might occur during drying and wilting of the grass, different drying methods were employed. Thus samples were dried in an oven at 95-100°C., wilted in the air, dried *in vacuo* over P_2O_5 and dried in a current of hot air.

When a current of hot air was employed, the dried grass contained essentially the same amounts of all non volatile acids as the fresh grass. By using the other methods, however, there were considerable losses in malic and citric acids, the losses being largest during the slow wilting process. With this in mind it was reasonable to believe that losses of malic and citric acids in silage were not due entirely to bacterial action, but also to the action of the plant enzymes.

The changes in the acid contents due to the plant enzymes were examined by analysis of fresh and wilted sterile timothy grass. Changes similar to those which occurred during wilting of rye grass were expected. However, in the microbe-free grass there was a loss of malate and a gain of citrate. Within the experimental errors these changes corresponded to the conversion of 1 mole malic acid to 1 mole citric acid. There was also an increase in quinic acid during the wilting. This, however, may possibly be an experimental error, as the amounts of grass analysed were relatively small.

Decrease in malic acid and increase in the citric acid content has been reported by Pucher et al. (96) to occur when excised tobacco leaves were cultured in the dark. The same workers found that both malic and citric acids disappeared when rhubarb leaves were incubated on water in darkness (97).

Wood et al. (98,99,100) examined the organic acids in Sudan grass, Kikuyu grass and Algerian oats. They found that

the behaviour of malic and citric acids was essentially the same in the leaves of all these when they were subjected to a gentle stream of air in darkness. The results, however, differed from those obtained with tobacco and rhubarb, as there was an increase both in malic and citric acids in the early stage of the starvation period. The content of the two acids reached a maximum and then decreased again at the end of the 7 day starvation period (99).

Wood et al. explained the conflicting results between grasses, tobacco and rhubarb by assuming differences in the individual enzyme activities.

Wilting of ryegrass and sterile timothy grass gave different changes in the malic and citric acid contents. Differences in enzyme activities due to different environments during the growing period might well be a possible explanation for this. The rye grass had grown naturally in the field while the sterile timothy grass had grown on a nutrient solution in test tubes in artificial light. Also it must be remembered that they are different species.

The results of the wilting experiments can not be compared strictly with the starvation experiments quoted above, as the differences in species and experimental techniques have to be considered.

The conversion of malate to citrate in the sterile grass, however, might be explained on the basis of the Krebs cycle, as malate could be converted to oxaloacetate which could combine with pyruvate to form a tricarboxylic acid (see fig.1, p. 3)

Silage with metabisulphite addition.

Sodium metabisulphite acts as a bactericide and has been used as such as an additive to silage. The changes occurring in a metabisulphite silage will therefore be due largely to the plant enzymes.

In a metabisulphite experiment carried out at the Edinburgh and East of Scotland College of Agriculture (Silage No.5), the non volatile acids were examined after 6 months ensilage. When metabisulphite had been added, the bacterial count was very small (101). The fresh grass, from which the silage was made, was not analysed. However, the grass was cut on May the 1st from the same plot as the grass analysed on April the 25th (see table 11). Although 5 days older it is unlikely in view of the evidence given above, that the organic acid content had changed significantly. Assuming this, there was an increase in the citric acid content during ensilage when metabisulphite was added alone and when both metabisulphite and lactobacilli were added. There was a decrease in malic acid, especially when inoculated with lactobacilli. There was no increase in succinic acid.

The effect of addition of metabisulphite on the behaviour of the non volatile organic acids can be seen by comparing these two silages with silage made from the same grass sample with addition of lactobacilli only. In this case a normal silage with a low pH was obtained and malic and citric acids had almost completely disappeared.

It is tempting to connect the increase in citric acid in this experiment with the increase observed during wilting of sterile grass which could then be explained as due to the action of the plant enzymes, although it must be remembered that the microorganisms were not entirely absent in the metabisulphite experiment. The results from the metabisulphite experiment also suggest that part of the malic acid in ordinary silage is broken down by action of microorganisms, and that the disappearance of citric acid is entirely due to bacterial action.

Rosenberger (102) found that Clostridium sphenoides strains (isolated from silage) attacked malate in pure culture and most of them also fermented citrate at about pH 7. Also some strains of Cl. butyricum were able to ferment malate. In pure cultures of lactobacilli, malate disappears from the medium with evolution of gas and rise of pH, suggesting a decarboxylation. It does not, however, appear to act as an energy source (103).

Succinic acid was never found to change significantly in

amount during wilting of rye grass or sterile timothy, nor was it found in silage when metabisulphite had been added. Otherwise it appeared in varied amount in silage. It is known that propionibacteria are able to produce succinic acid (9,104) from various substrates. Succinic acid may also appear by deamination of aspartic acid by coliform bacteria (104a).

However, the bacterial changes occurring in silage may depend upon many factors and it is not an easy matter to state the exact course or mechanisms of these changes. First of all the types of microorganisms and the numbers may differ from one sample of grass to another. Even if the sample from which the experimental silages are made is mixed well, the initial microflora may differ in different bottles. This may cause differences in the fermentation.

However, the familiar lactic acid fermentation of carbohydrate was observed in the silage experiments. Acetic acid, which can be produced by many organisms from a variety of substrates, was found in all silages. In Silage No.3 it was found to be present in a larger amount when citrate had been added than in the control silage. This may suggest a bacterial fermentation of citrate with acetate as the main product. Lactic acid was expected to appear in a silage with malate added. This was not the case however. Instead, the succinic acid figure was higher than in the control, although succinic acid need not necessarily come from malic acid.

Considering Silage No.2 (see figs 25 and 26) it appears that succinic acid could be an intermediate in the propionic acid formation. The succinic acid was formed very rapidly and did not increase much from a 2-day silage to a 1-week silage. If the figure for the 1-week silage is considered as the maximum, the disappearance of succinic acid would account for the propionic acid formed.

When this work was started it was not known to what extent organic acids contributed to the digestible components of grass, hay and silage. From the results obtained from analyses of perennial rye grass by accurate methods, it is seen that the total amount of organic acids is small in the fresh material. In silage the organic acids, mainly lactic and acetic, representing the main products in the fermentation of the carbohydrate originally present, are of considerable feeding value to the ruminants (18e,26,105).

The malic and citric acids which are lost in silage-making do not represent more than 2-3% of the dry matter. It is possible too that the products of this breakdown are digestible substances such as lower fatty acids, thus reducing the actual loss of feeding value.

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SUMMARY

1. Chromatography of non volatile organic acids on an anion exchange resin has been adapted to grass and silage analyses.

2. Volatile acids have been determined in silage by chromatography on silica gel.

3. Lactic acid has been determined colorimetrically in silage extracts purified by ion exchange resins.

4. In agreement with previous workers, quinic, malic and citric acids have been found to be the main acids in perennial rye grass. Malic acid increased in amount during the growing period but there was little change in quinic, citric and succinic acids.

5. Malic and citric acids disappeared rapidly during silage fermentation, while succinic acid appeared in varying quantities, the increase being highest in a high-pH silage.

These changes were greatly reduced by using metabisulphite as an additive to the silage.

6. During wilting and oven drying of the grass, there was a decrease in the amounts of malic and citric acids. Rapid drying in a current of hot air did not alter the composition of the non volatile acids.

7. During wilting of sterile grass, malic acid decreased and citric acid increased in amount.

8. The results are discussed in relation to reactions brought about by plant enzymes and microorganisms.

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